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REACTIONS OF THE PULMONARY AND BRONCHIAL BLOOD VESSELS

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It has long been recognised that the greatest obstacle to an accurate analysis of intrinsic pulmonary vascular mechanisms is their almost constant association with cardiac and systemic circulation phenomena. Definite information of the occurrence of such mechanisms can only be obtained by measurements of the pressure and flow of blood entering and leaving the lungs and of the blood content of the lungs, yet all these factors may undergo modification merely as a result of cardiac and systemic circulation variations. The earlier investigators who for the most part were aware of these difficulties approached the problem by making simultaneous observations on the entire animal of the pulmonary and systemic pressures during nerve excitations or the exhibition of drugs, active participation of the lung blood vessels being assessed by an analysis of the time relations and magnitude of the pressure changes in the two circulations. This work led to the growth of two schools of thought, the one which considered the pulmonary vascular system to be under a somewhat weak control of the nervous system, the other which denied any active participation of the pulmonary blood vessels in circulatory phenomena, the lesser circulation, apart from the influence of respiration, being regulated by changes taking place in the heart output and in the greater circulation. The protagonists of the latter school maintained that the experiments purporting to demonstrate the active control of the pulmonary blood vessels could be equally well explained by passive effects, a contention which gave rise to a series of investigations on isolated perfused lungs and isolated pulmonary vessels in the hope that experiments under more rigidly controlled conditions would definitely settle the question. Although this hope has not been fully realised with regard to a complete understanding of the nervous mechanisms con-

trolling the lung blood vessels, there is a general agreement between those who have analysed and summarised the available data in the past that such a control does exist. A history of the problem up to 1923 from the physiological and pharmacological aspects is to be found in the papers by Bradford and Dean (1889), Schafer and Lim (1919), Luckhardt and Carlson (1921), Schafer (1921), Wiggers (1921) and Tigerstedt (1923). Recent work on the subject shows that the reactions of the various parts of the pulmonary vascular bed to nervous or chemical influences are not always similar and that any given response of the lungs as a whole must be considered as the resultant of a number of reactions in different parts. Such a conception of the pulmonary vascular system is by no means new but it does not seem to have been sufficiently stressed and failure to recognise its truth may lead to errors in the interpretation of observations. Experiments on isolated perfused lungs and isolated pulmonary vessels have of necessity played a large part in the detailed examination of these reactions. But Nature forever ready to defeat an inquiry into her secrets has shown us that slight departures from her own conditions may introduce complications which require special attention. In the case of isolated lung preparations the complications introduced are presumably due to the abnormal perfusate which may be responsible for a diminished sensitivity of the preparation or for changes in the "background" of the tissue so that abnormal reactions to chemical and nervous stimuli may arise. It is desirable therefore in reviewing our knowledge of the reactions of the pulmonary blood vessels to inquire how far they are dependent upon the experimental conditions; it is only by this method that we can hope to know the normal reactions and the conditions which determine them, and at the same time be in a position to assess the value of such observations on the isolated organ or tissue preparation. A certain amount of overlapping with previous reviews will be necessary, nevertheless this will be avoided as far as possible by omitting or only mentioning briefly the investigations on the entire animal which have been fully discussed by the authors already cited.

Isolated perfused lungs. R. Lower (1669) may be said to have first used the method although it was not until Cavazzani (1891), Brodie (1903), Brodie and Dixon (1904) and Plumier (1904) carried out their investigations that it became generally adopted. Some physiologists believe that intrinsic pulmonary mechanisms can only be examined in this manner, others consider such perfusion experiments to be conducted on dying tissue and therefore too abnormal to yield information of processes occurring in the normal animal. That both sides overstate their

case is indicated by a fair agreement of results obtained by experiments on the isolated lungs and on the entire animal. Such investigations as those conducted by Schafer and Lim (1919) and Ettinger (1932, 1932a) on the effect of adrenaline on the pulmonary circulation are invaluable because observations on the entire animal under natural but not absolutely controlled conditions are reinforced by those on the perfused lungs under conditions which are less natural but on the whole better controlled. The advantages of the perfusion method are manifold; ventilation and perfusion pressure (or inflow) can be maintained constant so that alterations in blood inflow and outflow (or pressures) and blood volume of the lungs may be taken as a certain indication of intrinsic pulmonary phenomena. The method, however, suffers from the disadvantage that the perfusion fluid, whether it be Ringer's solution or a modified form of this solution, or blood rendered incoagulable by one of the usual procedures, is abnormal and in consequence the lung blood vessels are in general less sensitive in their response than they are in entire animals. All who have worked on the perfused lungs will have encountered this difficulty and indeed it has led to a special study of the conditions determining the response of the blood vessels with certain perfusates. Until these conditions are fully understood it will not be possible in all cases to decide how far the reactions are physiologically significant or dependent upon the production of an artificial "background" due to the abnormal perfusate. It is also true that it is not possible to apply the method to all animal species, the onset of oedema in some, especially with certain perfusates, preventing prolonged observations. Although these considerations are of fundamental importance they should not unduly weight the scales against the value of such experiments because many of the reactions of the pulmonary vascular bed can be readily reproduced with a variety of perfusion fluids and therefore the balance of probability is in favour of their being functionally significant.

Vasomotor nerves. *Activity in entire animals.* Previous to the work of Bradford and Dean (1889a, 1889b, 1894) it cannot be said that any convincing evidence was produced of the presence of a pulmonary vasomotor nerve control. These authors demonstrated that a rise in pulmonary arterial pressure in the dog could be produced by stimulation of the peripheral ends of cut thoracic nerves from D2 to D7 without cardiac acceleration or a systemic arterial pressure rise. Their observations were confirmed in the main by François-Franck (1895, 1895a) and Plumier (1904, 1905). Brodie and Dixon (1904) were also able to confirm most of the experimental results obtained by Bradford and Dean

but were unable to agree with them in concluding that the effects they observed could only be explained on the supposition that the pulmonary vessels were supplied with constrictor fibres and were therefore under nervous control (see also Dixon and Hoyle, 1928).

Activity in isolated perfused lungs. Brodie and Dixon failed to obtain any evidence of the functional activity of the vasomotor nerves from experiments conducted on the isolated perfused lungs of the cat. There is no doubt that their opinion carried great weight at the time and perhaps hardly less so now, if we may judge from the views expressed by many writers on the subject. One of their main criticisms, and admittedly a fair one, against the experiments on the entire animal was that an augmentation in cardiac output might be responsible for the raised pulmonary arterial pressure following excitation of the sympathetic nerves although the heart rate might remain constant. Opinion did not appear to be greatly influenced either by the work of Plumier (1904) who at about the same time obtained a pulmonary vasoconstriction in the isolated perfused lungs of the dog as a result of excitation of the *annulus Vieusenii* or by the attention drawn to the much earlier investigations of Cavazzani (1891) (on rabbit) who in a similar type of preparation found vasoconstriction and vasodilatation following excitation of the cervical vagus and cervical sympathetic nerves respectively. Tribe (1914) made it apparent that the sensitivity of the isolated preparation depended to a large extent upon the temperature and pressure of the perfusate and her careful researches have been largely responsible for the success of later workers in this field. She found in the cat that excitation of the stellate ganglion and its branches or of the sympathetic chain immediately below it led to a pure vasoconstriction or one followed by weak vasodilatation; in several experiments quite a marked vasodilatation was repeatedly obtained. She considered the pulmonary blood vessels to be supplied by constrictor and dilator fibres of sympathetic origin. In similar preparations Le Blanc and van Wijngaarden (1924) also obtained vasoconstriction in the cat on excitation of the stellate ganglion but vagus stimulation gave rise to vasodilatation; Löhr (1924) on the other hand, reported no effects on the blood vessels of the isolated perfused lungs following vagus stimulation in the cat. It was clear that the somewhat small changes in pulmonary flow or the absence of such changes following nerve excitations obtained in some experiments might be due to the lack of those conditions necessary to maintain the nutrition of the pulmonary vasomotor nerves, and therefore responsible for the view that they exert only a weak control. Per-

fused preparations are notoriously difficult to keep in good condition and the lungs are not exceptional in this respect. By simultaneous perfusion of the pulmonary and bronchial vascular systems with two separate pumps at constant outputs (Berry and Daly, 1931), Daly and Euler (1932) succeeded in maintaining the excitability of the pulmonary vasomotor nerves for three hours. They were led to use this type of perfusion because Berry, Brailsford and Daly (1931) who demonstrated bronchial arterial branches lying within the pulmonary nerve sheaths suggested that in the lungs perfused through the pulmonary artery alone the pulmonary nerves might receive an insufficient blood supply and in consequence lose excitability and conductivity, whereas additional perfusion of the bronchial arteries might serve to maintain the nerves in good condition. Daly and Euler showed that excitation of the stellate ganglion caused pulmonary vasoconstriction whereas stimulation of the cervical vagi and the cervical or thoracic vagosympathetic nerves produced strong vasoconstriction in some preparations but weak vasodilatation in others. Dogs were used and one of the more striking observations was the variable response of the pulmonary arterial pressure to excitation of similar nerve structures in the different preparations; one preparation would always give rise to a strong vasoconstriction on thoracic vagosympathetic stimulation and another invariably to a weak vasodilatation. Whether this result depended upon the variable number of constrictor and dilator fibres in the thoracic vagosympathetic nerve (Möllgaard, 1912; Braeucker, 1926) or to some difference in the state of the peripheral tissue or to both was not determined. In view of the finding by these workers that excitation of the thoracic vagosympathetic nerves may augment the mean effective pulmonary arterial pressure 40 per cent, a rise approximating to that obtained by occlusion of the main arterial branch to one lung, it would seem that the vasomotor nerves to the lungs may exert under certain conditions a profound effect on the pulmonary arterial pressure. If such profound changes occur normally the pulmonary vasomotor nerves may maintain a more or less constant pulmonary arterial pressure at all circulation rates, thereby preventing overloading the right side of the heart (see Shaw Dunn, 1919; Barcroft, 1925) or they may control the blood distribution within the lungs, a speculation not unsupported by experimental evidence, since it has been shown that in all probability the whole of the pulmonary vascular bed is not open except when the blood flow is very large. (Cohnheim and Litten, 1875; Toyama, 1925; Wearn, Barr and German, 1926; Wearn, Ernstene and German, 1928.) Stimulation of the vagosympathetic nerves gives rise

TABLE 1

Vasomotor effects in isolated perfused lungs

AUTHORS	ANIMAL	EFFECT	NERVE STRUCTURE EXCITED AND NATURE OF PERFUSATE
Cavazzani (1891)	Dog	V.D.	C.V.S.
Brodie and Dixon (1904)	Dog, cat, rabbit	0	A.V., I.C.G. or T.N., D.B. perfusate
Plumier (1904)	Dog	V.C.	A.V., D.B. perfusate
Daly and Euler (1932)	Dog	V.C. V.C. or V.D. sl.	S.G. C.V., C.V.S., or T.V.S. D.B. perfusate
Tribe (1914)	Cat	V.C., V.D. or V.C. → V.D. sl.	S.G. and sympathetic chain. D.B. perfusate
Le Blanc and v. Wijngaarden (1924)	Cat	V.C. V.D. → V.C.	S.G. C.V.
Löhr (1924)	Cat	0	C.V. Hirudinised blood
Cavazzani (1891)	Rabbit	V.D. V.C.	C.S. C.V.
Euler (1932)	Rabbit	0 V.C.	C.S. C.V., R.S. + D.B. perfusate
Krogh (1907)	Testudo Graeca	V.C.	V.S. R.S. perfusate
Luckhardt and Carlson (1921)	R. pipiens turtle	V.C. or V.D. sl. 0	C.V.S. C.S. R.S. + D.B. perfusate
Nisimaru (1922)	Toad, tortoise	V.C.	V.S. R.S. perfusate

TABLE 1—*Concluded*

AUTHORS	ANIMAL	EFFECT	NERVE STRUCTURE EXCITED AND NATURE OF PERFUSATE
Mashima (1921)	Toad (Japanese)	?V.C.	Vagus
		0	Sympathetic. R.S. perfusate
Fedotow (1932)	R. esculenta	V.C.	V.S.
		V.D. or V.C.	Spinal nerves 1 and and 2
		V.D. or V.C.	Vagus. R.S. persate

A.V. = Annulus Vieussenii. C.S. = cervical sympathetic. C.V. = cervical vagus. C.V.S. = cervical vagosympathetic. D.B. = defibrinated blood of same species. I.C.G. = inferior cervical ganglion. R.S. = Ringer solution. S.G. = stellate ganglion. T.N. = white rami of upper thoracic segments. T.V.S. = thoracic vagosympathetic. Sl. = slight. 0 = no effect. V.C. = vasoconstriction. V.D. = vasodilatation.

to vasoconstriction in the perfused lungs of Testudo Graeca (Krogh 1907, 1910), of Rana pipiens and turtle (Luckhardt and Carlson, 1921), and of the toad and tortoise (Nisimaru, 1922). According to Nisimaru the fibres responsible for this effect on the toad and tortoise are sympathetic in origin, arising from the 3rd and 4th spinal nerves to pass to the corresponding sympathetic ganglia, thence to the jugular ganglion and finally in the vagosympathetic trunk. (See table 1.) Fedotow (1932) working on a large number of R. esculenta obtained almost without exception a vasoconstriction on stimulating the vagosympathetic nerves. Excitation of pure vagal fibres above the point where the sympathetic joins them produced either a vasodilatation or constriction, the latter effect predominating. Stimulation of the sympathetic at the level of the upper two cerebrospinal nerves also led to either effect but vasodilatation predominated. The vasoconstrictor effect in each case weakened at the onset of autumn and during the winter. Weak stimulating currents acting upon the vagus were more effective than strong in producing a vasodilatation and in some experiments increasing the strength of current converted a vasodilatation to a mixed reaction and finally to a vasoconstriction. In order to control any possible error due to current spread, Fedotow excited the vagus with sodium chloride crystals and the sympathetic fibres by painting a nicotine solution on the first and second sympathetic ganglia. By these means of stimulation he confirmed his observations obtained by the electrical method.

The relation between bronchial and pulmonary-vascular phenomena. It has frequently been stated that the pulmonary vasomotor effects, as evidenced by changes in the blood flow following nerve excitations, are probably due to concomitant bronchomotor changes (Hess, 1930). The passage of some of the smaller pulmonary vessels and veins through the mesh of the bronchial musculature (Miller, 1925) and the presence of an interstitial musculature in the form of sheaths about the blood vessels (Baltisberger, 1921) suggest that contraction of either or both these muscle systems would not be without influence on the blood flow through the lungs. It is true that at the present time we have little knowledge of the functions of the interstitial system but it is not improbable that it is under nervous control. Mashima (1921), working on Japanese toads, perfused the lungs with Ringer solution and observed a diminution in outflow following the exhibition of adrenaline or vagus excitation. He considered the apparent vasoconstriction following vagus stimulation to be due to compression of the blood vessels by the contraction of the smooth muscle stroma tissue which also took place. Luckhardt and Carlson (1921) designed experiments to demonstrate the independence of tonus changes in the lung musculature and vasoconstriction which usually occur as a result of exciting the vagus in the frog. Both effects are eliminated practically at the same rate by atropine in the perfusion fluid but when a few drops of dilute atropine are painted directly on the pulmonary arteries, the vasomotor effects fail before the lung motor nerves. In the isolated lungs of the dog Daly and Euler (1932) were able to obtain in a few experiments well marked vasoconstriction following excitation of the cervical vagi or vagosympathetic nerves, the stellate ganglia or the thoracic vagosympathetic nerves, in the absence of bronchoconstriction, thus demonstrating that the vascular effects are able to occur independently of the bronchomotor. These observations, however, do not militate against bronchomotor effects having some influence on the pulmonary vascular resistance, they only show that resistance changes due to nerve excitation can occur independently of bronchial constriction. The part played by the interstitial musculature is unknown. The possibility that the type of vascular response to nerve excitation is dictated by the condition of nerve ganglia situated between the point of excitation and the periphery should be considered. Braeucker (1926) and Braeucker and Kemmel (1927) observed in the case of bronchomotor fibres that two successive stimulations of the same nerve, separated by a short interval of time, the current strength and duration being unaltered, may give rise to diametrically opposed effects on the

bronchial calibre. They considered this effect to be due to the changing response of the ganglia in the bronchial wall. It is possible that intrapulmonary ganglia situated on the course of the vasomotor nerves might act in the same way although the existence of such ganglia is at present doubtful (Larsell, 1921). It should not be forgotten, however, that ganglia of the pulmonary plexuses or of the larger bronchi may give rise to postganglionic vasomotor fibres, for Larsell has shown that the smaller arterial branches receive twigs from the nerve plexus around the smaller bronchi which accompany them.

ADRENALINE. *Action in the entire animal.* Large doses of adrenaline injected into the dog or cat generally produce a rise in pulmonary arterial pressure which has been ascribed to one or more of the following factors—"back pressure," active pulmonary vasoconstriction, increased venous flow to the heart, increased cardiac output, and unequal action of the two sides of the heart. Schafer and Lim (1919) who made a critical analysis of previous work, carried out experiments on entire animals (dog, cat and rabbit) and reached the conclusion that the rise of pulmonary arterial pressure after adrenaline was due partly to pulmonary vasoconstriction, and partly to changes in venous filling and output of the heart. They obtained no evidence that "back pressure" effects influenced the pulmonary arterial pressure, and described certain differences in the adrenaline response of the three species. In view of more recent investigations the interpretation of pulmonary vascular events in the entire animal in response to adrenalin injections has been made still more difficult. McDowall (1923) came to the conclusion that although adrenaline constricted the pulmonary vessels there may be an increased efficiency of the left ventricle. Dixon and Hoyle (1929, 1930) drew attention to the effect of adrenaline in raising the pulmonary arterial pressure by dilating the coronary vessels and increasing the flow to the right heart (Anrep and Bulatao, 1925). Berry and Daly (1931) showed that a rise in aortic pressure may lead to a rise in the pulmonary arterial pressure due to an increased blood flow from the bronchial to the pulmonary capillaries, thereby simulating "back pressure" effects; this mechanism therefore may come into play in the entire animal as a consequence of the raised systemic pressure due to adrenaline injections.

Action in isolated perfused lungs. A large number of investigators have conducted experiments on the effect of adrenaline on the isolated perfused lungs but the results even on animals of the same species are not always in agreement. It would appear that lack of uniformity may in part be explained by differences in the preparation and dosage of

TABLE 2

The effect of adrenaline on the blood vessels of isolated perfused lungs

AUTHORS	ANIMAL	EFFECT	PERFUSATE, ADRENALINE PREPARATION AND DOSAGE
Brodie and Dixon (1904)	Dog	D	D.B. B.W.T. or P.D. Ch 100γ
Plumier (1904)	Dog	C	D.B. Sr. 200γ
Wiggers (1909)	Dog	C	L.S. P.D. Cr. 20γ
Farini (1910, 1911)	Dog	C	L.S. P.D. or Merck F.d. = 1/150,000
Heger (1912)	Dog	0	L.S. 1000γ
Plumier Clermont (1912; 1912a)	Dog	C	L.S. + D.B. P.D. Cr. 2γ
Führer and Starling (1913)	Dog	C	D.B. P.D. Ch. . . . .
Tribe (1914)	Dog	C	D.B. P.D. Ch. or P.D. Cr. 10γ
Schlesinger (1931)	Dog	C or D sl. → C	D.B. (ox + dog) Sr. 2000γ
Daly and Euler (1932)	Dog	C	D.B. B.W.T. 20γ
Brodie and Dixon (1904)	Cat	D	D.B. B.W.T. or P.D. Ch. 50γ

TABLE 2—*Continued*

AUTHORS	ANIMAL	EFFECT	PERFUSATE, ADRENALINE PREPARATION AND DOSAGE
Wiggers (1909)	Cat	C.	L.S. P.D. Cr. 1000γ
Campbell (1911)	Cat	C. sl.	R.S. B.W. Hemisine 250γ
Tribe (1912, 1914)	Cat	C. → D. D.	D.B. P.D. Ch. = D.; P.D. Cr. or H. or S.E. 10γ = C → D: 0.02γ = D.
Modrakowski (1914)	Cat	C. or D.	D.B. . . . . F.d. = 1/17 × 10^5
Schafer and Lim (1919)	Cat	C. sl.	L.S. or L.S. + 6 p.c. G.S. P.D. Ch. F.d. = 1/60,000
Löhr (1924)	Cat	C. → D. D.	Hir. B. P. Ch. or Sr., F.d. 1/10^9 = D. 1/15 × 10^7 = C. → D.
Sakussow (1927)	Cat	C. or D.	R.S. . . . . F.d. = 1/10^6
Prawdins and Kusnezoff (1930)	Cat	C.	. . . . F.d. = 1/10^6
Brodie and Dixon (1904)	Rabbit	C. or D.	? D.B. B.W.T. or P.D. Ch. . . . .
Farini (1910)	Rabbit	C.	L.S. P.D. Ch. or Merck. F.d. = 1/500,000
Campbell (1911)	Rabbit	C. sl.	R.S. B.W.H. . . . .

TABLE 2—*Continued*

AUTHORS	ANIMAL	EFFECT	PERFUSATE, ADRENALINE PREPARATION AND DOSAGE
Tribe (1912, 1914)	Rabbit	C. or D.	Hir. B. P.D. Ch. = D. Other preps. = C., 10γ
Beresin (1914)	Rabbit	D.	L.S. M.L.B. Sr. or P.D. F.d. = $1/4 \times 10^6$
Schafer and Lim (1919)	Rabbit	C.	L.S. or L.S. + 6 p.c. G.S. P.D. Ch. F.d. = 1/80,000
Miyake (1930c)	Rabbit	C.	L.S. . . . .
Euler (1932)	Rabbit	C.	R.S. + 6 p.c. G.S. B.W.T. 10γ
Tribe (1912)	Guinea pig Rat	C. or D.	D.B. P.D. Cr. = C. P.D. Ch. = D. 10γ
Baehr and Pick (1913)	Guinea pig	O.	T.S. P.D. Ch. F.d. = 1/100,000
Ettinger (1929)	Guinea pig	C. or O.	T.S. Seasonal variation . . . .
Hirakawa (1925)	Rat	O.	R.S. Sankyo & Co. 100γ
Tribe (1914)	Ferret	C.	D.B. P.D. Ch., P.D. Cr. or H. 10γ
Tribe (1912)	Pigeon Frog Tortoise	C. or D.	P.D. Cr. or H. = C. P.D. Ch. = D.
Rothlin (1920)	Frog R. temp.	C. D.	R.S. > 1γ = C. < 1γ = D.

TABLE 2—*Concluded*

AUTHORS	ANIMAL	EFFECT	PERFUSATE, ADRENALINE PREPARATION AND DOSAGE
Luckhardt and Carlson (1921)	Frog R. pip.	C. D.	R.S. + D.B. $> 5\gamma$ = C. $< 5\gamma$ = D.
Adler (1921)	Frog R. esc.	C, D,	R.S. + H.S. Sr. 10γ = C; 0.001γ = D.
Michalewskaja (1930)	Frog	D,	R.S. . . . . F.d. = $1/10^6$
Mashima (1921)	Toad (Japanese)	C. or D.	R.S. 2γ = C.
Nisimaru (1922)	Toad	C.	R.S. 1000γ
Luckhardt and Carlson (1921)	Turtle	C. D.	R.S. + D.B. 200γ = C. 50γ = D.
Nisimaru (1922)	Tortoise	C.	R.S. 1000γ
Krawkow (1913)	Pike (gills)	D.	R.L.S. P.D. or l-Sr. (Höchst) F.d. = $1/10^7$
Fedotow (1932)	R. esculenta	V.D., V.C. or V.C. → V.D.	R.L.S.

C. = constriction. D. = dilatation. 0 = no effect. sl. = slight effect. D.B. = defibrinated blood of same species. R.S. = Ringer solution. L.S. = Locke solution. T.S. = Tyrode solution. Hir. B. = hirudinised blood. H.S. = horse serum. G.S. = gum saline. B.W.T. = Burroughs Wellcome and Co., Tabloid. P.D. Ch. = Parke Davis & Co., Adrenaline chloride, 1/1000 with 5 p.c. chloretone. P.D. Cr. = P.D. and Co., crystalline adrenaline. H. = Hemisine, B.W. and Co. S.E. = suprarenal gland extract. Sr. = suprarenin. M.L.B. = Meister, Lucius and Brüning. F.d. = final dilution. P.V. = pulmonary veins. l-Sr = laevorotary Sr.

The minimum effective dose is given in γ (= 0.001 mgm.) or as the final dilution.

adrenaline, the perfusion solution, the temperature and perfusion pressure (Tribe, 1914), the species of animal, and the toxic effect of the anesthetic used for induction. (Brodie and Dixon, 1903; Jackson, 1912; McDowall, 1923a.) The initial tonus of the blood vessels may also determine the response (Luckhardt and Carlson, 1921).

With such a large number of factors potentially responsible for the discrepancies it has been thought desirable to incorporate a table showing the main differences in experimentation of the various workers. In table 2 attention is directed towards the adrenaline response of the blood vessels in the isolated perfused lungs under different conditions of perfusion in the various animal species. Wherever possible, the type of adrenaline preparation and the minimum effective dose is indicated, either as the final dilution of adrenaline in the perfusate or as the amount injected into the perfusate as it enters the lungs.

1. *Adrenaline preparation.* The only question which need be considered here is the effect of 0.5 per cent chloretone present in a much used adrenaline preparation (Parke Davis & Co. 1:1000).

Tribe (1912) in a preliminary communication stated that in the isolated lungs of rabbits, rats, guinea pigs, pigeons, snakes, tortoises and frogs, adrenaline with chloretone produces intense vasodilatation whereas crystalline adrenaline or Hemisine (P.D.) causes vasoconstriction. Cats' lungs perfused with defibrinated blood give the same results. She attributed the vasodilator effects obtained by Brodie and Dixon (1904) with large doses of adrenaline on the cat, partly to the presence of chloretone in their preparation, but in a later paper (1914) considered their effects were mostly due to a high pressure and a low temperature of the perfusion fluid. It should be noted, however, that Brodie and Dixon (1904) in their first experiments used adrenaline (B.W. tab.) and in the later experiments the preparation containing chloretone, much more uniform results being obtained with the latter preparation although they make no mention of a marked difference in action between the two, or which preparation was used in any given experiment. In this connection Löhr (1924) obtained the same results in the cat, using either the adrenaline-chloretone preparation or suprarenin Höchst, and fresh or old blood or Locke solution as a perfusate made no difference to the effect. Moreover Schafer and Lim (1919) in the rabbit and cat never observed dilatation with the adrenaline chloretone preparation, only constriction. Berry and Daly (1932) were unable to detect any difference between adrenaline preparations with and without chloretone on the isloated dog's lungs perfused at normal pressure and temperature.

2. *Dosage.* Small doses of adrenaline have been found to produce vasodilatation and large doses vasoconstriction, in the cat (Tribe, 1914); in the frog and turtle (Luckhardt and Carlson, 1921); in the frog (Rothlin, 1920; Adler, 1921) and in the toad (Mashima, 1921). Modrakowski (1914) found constriction or dilatation in the cat with all doses ranging from 0.06 to 0.3 mgm. in 100 cc. of defibrinated blood and Sakussow (1927) reported in the same sense. Löhr (1924) in one cat observed increasing doses of adrenaline to convert a pure dilatation to constriction succeeded by dilatation. With large doses of adrenaline (2–5 mgm.), Schlesinger (1931) obtained on some isolated lung preparations of the dog, a slight vasodilatation followed by constriction. It is conceivable that such a response may be due to the arrival at the site of action of a weak solution of adrenaline followed by a strong one owing to a mixing in the blood stream as suggested by Fleisch (1931). Schlesinger used perfusion pressures of 60 to 80 mm. Hg.

3. *Perfusion pressure and temperature.* Tribe (1914) found that at low perfusion pressures, the immediate effect of the adrenaline chloretone preparation is vasoconstriction followed by dilatation, whereas at high pressures even with a dose of 0.2 mgm. only vasodilatation is present. The explanation put forward was that the weak constrictor action of this preparation is unable to overcome the passive dilatation of the vessels at high pressures, and thus only the dilator effect of the adrenaline is obtained. With regard to temperature, a fall from body to room temperature would often reverse the constrictor effect of large doses of adrenaline to one of vasodilatation. In this connection Beresin (1914) always obtained vasodilatation in the rabbit with three different preparations of adrenaline with the temperature of the perfusion fluid at 20°C. whereas in the same species of animal Campbell (1911), Schafer and Lim (1919), Miyake (1930c) and Euler (1932) obtained vasoconstriction, with moderate or low perfusion pressures and normal temperatures. Tribe suggested that the absence of constriction at low temperatures was due to the pulmonary arterioles being already constricted from cold and therefore unable to undergo further constriction. Her results may be summed up by saying that high pressures and low temperatures tend to eliminate the vasoconstrictor action of adrenaline leaving only the vasodilator action, but this effect is more easily seen when using the adrenaline-chloretone preparation which has a weaker constrictor action than other preparations; at normal perfusion pressures and temperature large doses of adrenaline produce a true vasoconstriction, and small, a vasodilatation. Heger (1912) (on dog) using high pressures for perfu-

sion (52 cm. H_2O) obtained negative results with 1.0 mgm. adrenaline. Michalewskaja (1930) in 10 out of 14 experiments on frog's lungs procured definite evidence that the vasodilator effect of small concentrations of adrenaline was much greater at high than low perfusion pressures, but in 3 experiments the adrenaline effect was greater at the lower perfusion pressures. On perfusing the liver he found that the vasoconstrictor response to adrenaline showed the reverse relationship because with the lower perfusion pressures the vasoconstriction was greater. Stypticin and KCl added to the lung perfusate brought about a vasoconstriction which was more marked at low than high pressures. This author quotes Prawdins and Kusnezoff (1930) as finding the vasoconstriction of the pulmonary vessels due to adrenaline, eserine or barium chloride or the vasodilatation due to caffeine to be larger at the higher pressures. Swetschnikoff (1913) failed to detect any very definite relation between the perfusion pressure and constrictor action of adrenaline, likewise Berry and Daly (1932) working on the isolated perfused lungs of the dog, although there was a slight tendency for the constrictor to be larger at high than low pressures in a few experiments. The latter investigators observed in one experiment that acetyl choline caused vasoconstriction at low and vasodilatation at high pressures, an effect which could be obtained repeatedly. In a second experiment acetyl choline always caused constriction but this was much greater at the lower perfusion pressures.

It is probable that the differences in the response to drugs, of the pulmonary vascular system perfused at various pressures may be explained in part by the fact that at the higher pressures a larger vascular territory will be perfused than at the lower. If this is the case the enhancement of drug action observed at high perfusion pressures will depend upon the increased vascular bed acted upon. This mechanism, however, will not account for all the observations. Apart from the possibility that a rise in perfusion pressure may alter, by virtue of stretching the walls, the reaction to drugs of blood vessels already open, the problem is additionally complicated owing to the evidence that all parts of the pulmonary bed do not respond to drugs in the same manner, and if such an augmentation of vascular territory does occur on raising the perfusion pressure, it is not safe to assume that the newly opened vessels have exactly the same pharmacological reactions as those already open. It might well be that the relation between perfusion pressure and the response to drugs (and to excitation of the vasomotor nerves) of the isolated perfused lungs as a whole may depend in part upon whether the action of the drug on the newly opened vessels is predominantly the same or oppo-

site to that on the vessels initially open; if the same, a rise in perfusion pressure will cause enhancement of the action; if opposite, a diminution or reversal of the initial action.

4. *Perfusion fluid.* An examination of the table reveals little evidence that the nature of the perfusion fluid influences the reaction of the pulmonary blood vessels to adrenaline. Indeed, Löhr (1924) states that his results with Ringer solution or defibrinated blood, fresh or old, used for perfusion were similar. Perfusion of rabbit's lungs with defibrinated blood obtained from animals of the same species and mixed with the blood of the animal from which the preparation is made, leads to such marked vasoconstriction that the pulmonary flow is negligible or absent (Tribe, 1914; Euler, 1932, and others), and under such conditions it is not possible to test the effects of adrenaline. It has been shown that the response to adrenaline of the systemic peripheral circulation may be determined by the hydrogen ion concentration of the perfusate (Kretschmer, 1907; Allday-Redonnet, 1920; Snyder and Campbell, 1920; Schmidt, 1921; Hulse, 1922), by the concentration of calcium or potassium salts therein (Pearce, 1913; Allday-Redonnet, 1920; Hulse, 1922; Voss, 1926), and by the initial concentration of adrenaline in the blood (Bauer and Fröhlich, 1919; Burn, 1932). As Lovatt Evans (1926) points out, the subject of the action of various ions is in a chaotic condition. True as this may be, investigations on these lines carried out in relation to pulmonary blood vessels would be of great interest although as far as can be ascertained little work in this direction has been carried out. It is of interest to note that recent work by Schretrenmayr (1931) indicates that the action of calcium on vascular tone is qualitatively comparable to that of adrenaline. Wiggers (1909) drew attention to the part played by the viscosity of the solution used for injection of the adrenaline and showed that if it is of less viscosity than the perfusion fluid then the "viscosity dilatation" might counterbalance the adrenaline constriction unless the concentration of the drug is very large. The necessity for control experiments to eliminate an effect of this kind is of importance.

5. *Tonus of blood vessels.* In the turtle Luckhardt and Carlson (1921) observed that the weakest concentration of adrenaline that affects the pulmonary blood vessels at all causes vasodilatation, but if the vessels are in marked tonus the vasodilatation caused by the minimal quantities of adrenaline may increase the rate of the blood flow through the lung several hundred per cent. From the work of Tribe (1914) it seems that the increased tonus of the blood vessels due to a temperature below normal is also released by adrenaline.

6. *Species.* In the dog, adrenaline has been found to produce vasoconstriction (Plumier, 1904; Wiggers, 1909; Farini, 1910; Plumier-Clermont, 1912, 1912a; Führer and Starling, 1913; Tribe, 1914; Schlesinger, 1931; Daly and Euler, 1932) or vasodilatation (Brodie and Dixon 1904) or vasodilatation followed by vasoconstriction (Schlesinger, 1931): in the rabbit, vasoconstriction, (Farini, 1910; Campbell, 1911; Tribe, 1914; Schafter and Lim, 1919; Miyake, 1930c; Euler, 1932), or vasodilatation (Brodie and Dixon, 1904; Beresin, 1914). In the cat adrenaline may give rise to vasoconstriction (Campbell, 1911; Wiggers, 1909; Schafer and Lim, 1919) or vasodilatation (Brodie and Dixon, 1904); or either effect, depending on the dose (Tribe, 1914; Löhr, 1924) or irrespective of the dose (Modrakowski, 1914; Sakussow, 1927). The table suggests that vasodilatation is more easily obtained in the cat than the dog and recent work by Gaddum and Holtz (1932) supports this view. The relative sensitivity of the different species to adrenaline is difficult to assess when the results are collected from various workers but Schafer and Lim (1919) reported that the pulmonary vessels of the cat are far less sensitive than those of the rabbit; this statement relates to the vasoconstrictor response of the preparations perfused with Ringer solution and gum saline, and it is to be noted that they never recorded a vasodilator response in the cat or rabbit. There is general agreement that the pulmonary vascular system is less responsive to adrenaline than the systemic circulation but until we have further evidence of the conditions which determine the sensitivity of perfused lungs to adrenaline no quantitative comparison is possible.

7. *Seasonal variations.* Luckhardt and Carlson (1921) noted that frogs kept in running water (8°–10°C.) during the month of December exhibited as a whole much feebler vasomotor responses in the lungs than the frogs worked on during the summer. By resorting to the expedient of slowly warming the frogs to approximately room temperature (20°–25°C.) before the experiments they increased the intensity and rapidity of pulmonary vasomotor reaction. Ettinger (1929, 1931a) working on male and non-pregnant female guinea pigs observed a periodicity of adrenaline reaction which seemed to be seasonal but varied in degree with the breed or climate. In Edinburgh a pulmonary vasoconstrictor action occurred from the spring to late autumn, but in Kingston, Ontario was limited to the period from late April to 14th June. In this connection a seasonal variation in the activity of the sympathetic portion of the autonomic system has been demonstrated by Cori (1921) McDowall (1931) and Armitage, McDowall and Mathur (1932).

8. *Interpretation of observations.* In assessing the effect of adrenaline on the isolated perfused lungs, most workers have used Brodie and Dixon's method of measuring the rate of outflow under a constant head of perfusion pressure. With this arrangement a change in outflow indicates the algebraical sum of the resistance variations taking place in the different parts of the vascular bed but gives no information of the territories affected or of the component resistance changes which determine the final outflow. Since transient outflow variations may be caused either by resistance or by blood capacity effects due to squeezing blood from the vessels, the method does not discriminate between these two phenomena during the initial and final stages of outflow variations, although a lasting alteration in outflow accurately indicates the total resistance change. It is clear that blood capacity phenomena in determining the outflow in the method under consideration can not be neglected and may be a source of error in the interpretation of the experiments, the more so as the lung vascular system as a whole may take several minutes to adjust itself to new conditions of blood flow brought about by altering the inflow, the venous resistance or the degree of inflation by negative pressure. Laux (1930) observed with increasing perfusion pressure the outflow to be smaller than the inflow. He used dogs' and cats' lungs under negative pressure ventilation. Recently some of these difficulties have been overcome by Gaddum and Holtz (1932) who recorded changes in the blood capacity of lungs perfused with defibrinated or heparinized blood, and drew the following conclusions as to the site of action of drugs on the pulmonary vessels. The constrictor effect of adrenaline in the dog was mostly due to an action on the arteries. Dilator effects which could only be demonstrated with certainty after ergotoxine were predominantly venous.

In the cat, both dilator and constrictor effects were seen in different experiments and at different stages of the same experiment; in each case the effect was partly arterial and partly venous in origin.

Action on isolated pulmonary vessels. Adrenaline causes contraction of the main pulmonary aorta and of the extrapulmonary arterial branches (Meyer, 1906; Langendorff, 1907; Dixon and Halliburton, 1910; Cow, 1911; Campbell, 1911; Barbour, 1912; Macht, 1914, 1915; Rothlin, 1920; Franklin, 1925, 1932; Miyake, 1930a, b; and Wissler, 1931). The intrapulmonary arteries are stated to be irresponsive to adrenaline by Cow (1911) but Röthlin (1920) reported a small relaxation effect, and Franklin (1932) very small and variable responses with contraction predominating. Wissler, (1931) observed adrenaline to

TABLE 3

The effect of adrenaline on isolated pulmonary blood vessels

AUTHORS	ANIMAL	EFFECT	REMARKS
Dixon and Halliburton (1910)	Dog	C.	P.A. P.D. Ch, P.D. Cr. or synthetic adr. M.L.B.
Waterman (1930)	Dog Cat	C.	Extra- and Intrapulm. V. P.D.Ch. F.d. = $1/7 \times 10^7$
Franklin (1932)	Dog	C.	Extrapulm. A. and V. P.D.Ch. and Cr. R.S. F.d. = $1/10^7$
Langendorff (1907)	Cat Sheep Pig, calf	C.	Extrapulm. A. R.L.S.
Campbell (1911)	Rabbit	C.	P.A. R.S. B.W.H.
Cow (1911)	Rabbit Sheep	C.	Extrapulm. A. R.S. F.d. = 1/1000
Barbour (1912)	Rabbit	C.	P.A. branches. R. S. Sr. Höchst. F.d. = 1/200,000
Franklin (1926)	Rabbit	C.	P.A. R.S. F.d. = 1/50,000
Meyer (1906)	Calf	C.	Extrapulm. A. R.S. Sr. Höchst. F.d. = $1/10^9$
Macht (1914)	Pig Ox Man	C.	P.A. br. L.S. F.d. = $1/10^6$
Barbour (1912)	Calf	C. (sl)[1] C. or D.[2]	1 = large, 2 = small Extrapulm. A. R.S. Sr. Höchst. F.d. = $1/2 \times 10^5$

TABLE 3—*Concluded*

AUTHORS	ANIMAL	EFFECT	REMARKS
Rothlin (1920)	Ox Horse	C.	Extra- and intrapulm. A. R.S. F.d. = $1/2 \times 10^6$
Wissler (1931)	Ox	C.[1] D.[2]	1 = large, 2 = small P.A. br. Pure crystals.
Campbell (1911)	Sheep	C.	P.A. and P.V. R.S. B.W.H. F.d. = $1/10^5$
Macht (1915)	Pig	C.	P.A. L.S. F.d. = 1/10,000
Barbour (1912)	Pig	D. or 0.	P.A. medium br. R.S. F.d. = 1/200,000
Miyake (1930a, b)	Ox	C.	P.A.

P.A. = pulmonary artery. A. = arteries. V. = veins. br. = branches. Other abbreviations as in previous table.

cause contraction of the central arteries and relaxation in some of the peripheral vessels with an intermediate indifferent zone. Barbour (1912) mentioned that he found a dilator response to adrenaline in a few of the smaller vessels tested.

Campbell (1911) found adrenaline to produce contraction of the pulmonary veins of the sheep and likewise Waterman (1930) in the dog and cat. Franklin (1932) obtained in the dog a contraction or no response, never relaxation of the large extrapulmonary veins; always contraction of the smaller extrapulmonary veins, and very small responses or none at all of the intrapulmonary veins, the relaxations balancing in number the contractions.

There appears to be general agreement that adrenaline contracts the extrapulmonary portions of the whole pulmonary vascular system in all species of animals tested (table 3) and the intrapulmonary vessels although less responsive tend to react by contraction or relaxation. The thorough investigation carried out by Franklin on the dog supports this

view, furthermore the adrenaline contraction effects he obtained were reversed by ergotoxine indicating the presence of sympathetic vasodilator mechanisms in addition to the sympathetic vasoconstrictor ones. He also brings forward suggestive evidence that sometimes, although rarely, the sympathetic vasodilator response may predominate over that of the sympathetic vasoconstriction, the concentration of the adrenaline not determining the type of reaction.

Microscopical observations on pulmonary circulation. Hall (1925) observed the circulation of blood in the smaller vessels of the lung with the aid of a microscope, using a transillumination method. He worked with cats and rabbits. In spite of the fact that the injection of 1 to 3 cc. of a 1:50,000 solution of adrenaline caused a cardiac acceleration, a definite decrease in the size of the larger and smaller arterioles was observed. R. G. MacGregor (1932) working in this laboratory using Hall's method has been unable to confirm the diminution in vessel size following the injection of adrenaline. The vessels examined ranged from capillaries to those having a diameter of 170μ, and the strength of adrenaline solution injected was the same or stronger than that used by Hall. Since Hall prepared his animals with chloretone and urethane or brief anesthesia followed by pithing whereas MacGregor anesthetised with urethane, luminal or medinal, the difference in result might depend upon the choice of anesthetic. Hall found that excitation of the vagi with concomitant slowing of the heart caused stoppage of the blood flow in the arterioles then a reversed flow, followed later by an onward flow and no capillary pulsation at each cardiac cycle was observed. MacGregor was able to confirm these findings. MacGregor extended his observations to the isolated cat's lungs perfused with the animal's own defibrinated blood by a constant output pump and observed adrenaline to cause a slowing of the blood stream, or sometimes a complete reversal (3 experiments) or an increase in blood flow (1 experiment) in the larger and smaller arterioles. Even so, no change in calibre of the blood vessels was detected although carefully sought for because it was expected that internal pressure alterations would produce a recognisable effect on the vessel diameter. The observations on complete reversal of flow with adrenaline were striking for in some vessels examined the reverse flow velocity equalled the initial flow, suggesting a marked change in resistance of one or more of the collateral vessels.

In cats under amytal anesthesia, Wearn, Barr and German (1926) exposed the lower lung lobe by removing the chest wall as far as the parietal pleura. A similar window was prepared through the diaphragm, the

parietal pleura being left intact. A beam of light was thrown through the diaphragm window and microscopical observations of the lung surface made through the thoracic window. A varying number of capillaries were seen to be open and their diameters varied greatly in size; moreover pressure on the abdomen opened up new capillaries. Wearn, Ernstene and Bromer (1928) counted in the majority of their experiments 6 capillaries or more to each air sac but in some instances there were but one or two capillaries per sac. They observed that adrenaline injected intravenously into the animal opened up new capillaries simultaneously with the rise in systemic arterial pressure.

ACETYL CHOLINE. *Action in the entire animal.* In the entire animal it is difficult to determine the action of acetyl choline on the pulmonary vascular system owing to concomitant effects on the heart and peripheral systemic circulation (Bennati, Gautrelet and Halpern, 1930). Antoniazzi (1932) studied the effect of acetyl choline in rabbits with the aid of Krogh's Chinese ink method. He concluded that this drug produced dilatation of the arterioles and constriction of the capillaries, effects which were inhibited by atropine.

Action in isolated perfused lungs. In the isolated perfused lungs, of the rabbit, cat and guinea pig, Hunt (1918) found that large doses added to Ringer's solution reduced the outflow, an action not abolished by atropine. Euler (1932) on the other hand, perfusing rabbits' lungs with a mixture of defibrinated blood and gum saline, observed a strong rise in the pulmonary arterial pressure with a greatly diminished outflow. This vasoconstrictor action was enhanced by eserine and suppressed by atropine. In the dog's isolated lung acetyl choline causes in some animals a vasoconstriction, in others a vasodilatation, both effects being enchanced by eserine and suppressed by atropine (Daly and Euler, 1932; Berry and Daly, 1932). Gaddum and Holtz (1932) observed that large doses of acetyl choline (0.1 mgm.) constricted the arteries of dogs, but small doses (3γ) produced vasodilatation in cats, the site of which was not determined. After larger doses (10–20γ) this vasodilatation was followed by constriction of the veins.

Action on isolated lung blood vessels. Franklin (1932) (on dog) obtained a contraction of all the extrapulmonary veins but relaxation of all the extrapulmonary arteries. He pointed out that the effect of acetyl choline, other things being equal, would be a pulmonary congestion. Waterman (1930) reported no affect with acetyl choline in three experiments on pulmonary veins. MacGregor (1932) observing with the aid of a microscope the blood vessels at the edge of the perfused lung

of the cat, noted an increase in blood flow in response to acetyl choline injections. That discrepancies may occur between conclusions drawn from experiments on isolated loops of vessel and those drawn from the perfusion of the isolated lungs is shown by the work of Gaddum and Holtz who in the latter preparation found that acetyl choline diminished the lung volume of the dog. It is not easy to reconcile this observation with Franklin's finding that acetyl choline dilates the arteries and constricts the veins. This discrepancy might be due to the difference in the media in which the vessels were bathed, or to the fact that Franklin's experiments were performed on animals killed by the humane killer, and those of Gaddum and Holtz on preparations from animals bled under anesthesia. In this connection it has been shown by Teschendorf (1921) that a small excess of hydroxyl ions in the fluid perfusing the frog's systemic circulation reverses the normal constrictor effect of acetyl choline to one of dilatation. In the same preparation Voss (1926) found acetyl choline to cause vasoconstriction if the perfusion fluid contained an excess of calcium ions, but vasodilatation if an excess of potassium ions, both effects being suppressed by atropine. At the beginning of an experiment acetyl choline produced vasoconstriction but at the end, vasodilatation; also an increase in the hydroxyl ion concentration of the perfusate increased the sensitivity of the preparation to acetyl choline. The literature relating to the actions of acetyl choline has been recently summarised by Riesser (1931).

Pharmacodynamic aspects of vasomotor action. It has sometimes been assumed that excitation of the thoracic sympathetic chain or stellate ganglion will give rise to pulmonary vasomotor effects which are pharmacologically sympathetic in nature whereas stimulation of the cervical vagus will produce effects pharmacologically parasympathetic. That such an assumption may be unwarranted is shown by the work of Euler and Gaddum (1931) who found that certain fibres running in the cervical sympathetic and having their cell station in the superior cervical ganglion are pharmacologically parasympathetic but anatomically sympathetic. In this connection, direct tests to determine the relation between pharmacological reactions and anatomical distribution of the pulmonary nerves are of interest. The pulmonary vasoconstrictor fibres running in the vago-sympathetic nerves appear to belong to the parasympathetic system in the frog and turtle (Luckhardt and Carlson, 1921) since their action is suppressed by atropine. The same is true for the constrictor fibres to the rabbit's lung, for Euler (1932) was not only able to show that excitation of the cervical vagi caused constriction

which was enhanced by eserine and suppressed by atropine, but also the constriction due to acetyl choline was similarly affected, this pharmacodynamic train of events indicating their parasympathetic nature (Loewi, 1921; Loewi and Navratil, 1926; Dale and Gaddum, 1930). Pharmacological evidence of a similar kind was produced by Berry and Daly (1932) in support of the presence of parasympathetic vasoconstrictor fibres in the thoracic vagosympathetic nerves in the dog and by Daly and Euler (1932) of parasympathetic vasodilator fibres running in the same nerves. The latter workers also obtained evidence of the presence of sympathetic vasoconstrictor and dilator fibres in the thoracic vagosympathetic nerves in the dog by showing that the constrictor effect consequent upon their excitation was suppressed or reversed by ergotamine tartrate, (Dale, 1906, 1913). In the cat Tribe (1914) observed in the isolated perfused lungs a slight constriction, or slight constriction followed by a small dilatation or a well marked dilatation following excitation of the stellate ganglion. Moreover, adrenaline gave rise to vasodilatation and not the usual vasoconstriction if the animal from which the preparation was made had been previously injected with 10 to 15 mgm. of ergotoxine phosphate. The position may be summarised by saying that the available pharmacological evidence obtained from observations on isolated perfused lungs and isolated vessels suggests that in the dog the lungs are supplied with sympathetic vasoconstrictor and dilator fibres as well as parasympathetic vasoconstrictor and dilator fibres. It would appear that the distribution within the lungs or the relative control exerted by each type of vasomotor nerve differs in the various species, so that it is not safe to assume that the observations made on one species will be repeated on another. Recently Ken Kuré and his collaborators (1931) have described vasoconstrictors for the pulmonary vessels in the dog which arise from spinal parasympathetic fibres in the dorsal spinal roots. Further investigations are necessary before their presence is proved (McSwiney, 1931).

THE BRONCHIAL VASCULAR SYSTEM. *Anatomy.* The bronchial arteries arise from the aorta, one or more intercostal arteries and the internal mammary artery, but their origin is variable (Haller, 1756; Krause, 1868; Nakamura, 1924). Wide differences between the distribution of the bronchial arteries within and on the surface of the lungs in various species have been demonstrated (Miller, 1906, 1908; Backman, 1923) and are especially marked in respect of the blood supply to the *pleura pulmonalis.* The bronchial arteries have long been recognised as nutritional to the bronchial tree (Reisseissen and Sömmering, 1808; Reisseis-

sen, 1822; Luschka, 1863; Konigstein, 1903) although as early as 1654, Marchetti described them as a separate blood supply to the lungs, and thus anticipated Ruysch (1771) who is sometimes given credit for the discovery.

Miller (1925, 1925a) found that the bronchial arteries supply the bronchial tree as far as the small bronchioles, but with the appearance of alveoli along the walls of the bronchioles, the bronchial arteries as a distinct set of vessels disappear, the terminal network of capillaries formed by them uniting with that of the pulmonary artery. Le Fort (1858), Luschka (1863), Küttner (1878) and Zuckerkandl (1881) stated that part of the respiratory capillary network is supplied by branches from the bronchial arteries. Whether the capillaries are the only channels of communication between the pulmonary and bronchial vascular systems or the smaller arteries and veins of each system also communicate with one another has been a much debated problem. This question has been discussed in detail by Berry, Brailsford and Daly (1932) and the weight of evidence appears to favour the opinion that in man and dog the capillaries are the only means of communication between the two systems. It is sufficient to mention here the work of Miller (1900, 1906a, 1925) in support of this view in which attention is drawn to the possibility that the *vasa vasorum* from the bronchial arteries to the branches of the pulmonary artery may be mistaken for anastomotic branches.

According to Miller (1925) the blood from the bronchial artery supplying the hilum with its adjacent pleura and the first two dividing points of the bronchial tree drains into the azygos, hemi-azygos or one of the intercostal veins, whereas that supplying the remaining bronchi and bronchioli as far as the capillary network in the walls of the *bronchioli respiratorii* empties into small branches of the pulmonary vein. An additional list of references to the anatomy of the bronchial vascular bed will be found at the end of this paper.

Interchange of blood between the pulmonary and bronchial vascular systems. Ghoreyeb and Karsner (1913) from injection experiments concluded that as long as a definite pressure is maintained in either the pulmonary or bronchial circulations the admixture of blood is extremely limited, although they thought it conceivable that more mixture may occur normally than under the conditions of their experiments. If, however, the pressure in either system sinks to zero the possibility of supply by the other system becomes evident. Berry and Daly (1932) found that the bronchial arterial inflow in two experiments was 18 and 5 per cent of the combined bronchial and pulmonary inflow. They carried

out simultaneous perfusion of the pulmonary and bronchial vascular systems including a small length of the esophagus. Rather more blood passed from the bronchial arteries to the pulmonary veins than to the bronchial veins provided the pulmonary and bronchial arterial pressures were within normal limits, but reducing the pulmonary arterial pressure to zero increased the proportion of blood passing to the pulmonary veins.[1] Raising the bronchial arterial pressure by increasing the pump output produced in some experiments a rise, in others a fall of pulmonary arterial pressure.

Vasomotor nerves. Nerves accompanying the bronchial arteries have been described by Reisseisen (1822), Kölliker (1852), Berkley (1893), Miller (1918) and Larsell (1922). Hovelacque (1927) states that the sympathetic twigs in man which run with the bronchial arteries arise usually from the sympathetic ganglia D3 and D4 (see also Rjasanskij, 1928). Miller described anastomosing nerve plexuses surrounding the bronchial arteries and bronchi, and Berry, Brailsford and Daly demonstrated the presence of bronchial arterial twigs within the sheaths of the nerves which accompany the main bronchial arteries. Thus, there is an intimate relation between the bronchial arteries and pulmonary nerves. In isolated lungs perfused through the bronchial artery, excitation of the thoracic vagosympathetic nerve or the injection of adrenaline gives rise to an increase in bronchial arterial pressure, the vasoconstriction due to the adrenaline being suppressed by ergotamine (Daly and Euler, 1932). There appears therefore to be little doubt that the bronchial vascular system is supplied with sympathetic vasoconstrictor fibres.

Pathology. Virchow (1847, 1851, 1856) found that occlusion of the pulmonary artery supplying one lobe leads to the development of a collateral circulation between the aorta and lungs by means of an increased development in the bronchial and intercostal arteries. The enlarged bronchial arteries were seen to communicate with branches of the intercostal arteries penetrating into the lung lobe, and an extensive network of vessels formed by the bronchial and intercostal arteries existed in the pleura. Gangrene of the lobe did not develop, the increased bronchial and the intercostal circulation being sufficient for the nutrition of the lobe. Following ligature of the pulmonary artery to one lung Schlaepfer (1924) reported an increased bronchial arterial supply to the same side with fibrosis of the lung. A different result is obtained if there is obstruction in a small branch of the pulmonary artery, for Kars-

[1] Line 4, page 332 of their paper should commence "the pulmonary arterial pressure zero and aortic pressure," etc.

ner and Ash (1912) have shown that under these conditions there is a lack of serious disturbance to the circulation due to the rich capillary and other anastomosis of the pulmonary artery itself rather than by anastomosis with the bronchial arteries. Moreover they observed no interference with nutrition of the bronchi up to a period of five weeks following ligation of the bronchial arteries thus demonstrating that the pulmonary circulation under certain conditions is sufficient to provide for the nutrition of the bronchi. Karsner and Ghoreyeb (1913) extended their observations to artificially produced pulmonary embolic areas involving less than an entire lobe. They concluded that no significant mixture of pulmonary and bronchial bloods takes place in these regions and with normal pressures in the two vascular systems these areas receive their blood supply almost entirely from the rich anastomosis of the pulmonary artery between its own branches. Holman and Mathes (1929), however, obtained evidence of a marked dilatation of the bronchial arterial branches leading to embolic areas which had been produced by the introduction of sterile or infected shot. A comparison of the bronchial arterial enlargement in vessels leading to sterile and infective emboli showed that the vessels to the infected area suffered greater enlargement than those to the sterile areas. Increased development of the bronchial arterial tree is also seen as a result of pulmonary stenosis and atresia (Christeller, 1916) and in the region of tuberculous lesions (Guillot, 1845). Miller (1925) has pointed out that the transitional zone from the bronchial to the pulmonary circulation in the bronchial tree is a favourite place for tubercles to develop and Forssman (1920) and Schmidt and Barth (1924) have drawn attention to the sensitivity of the bronchial vascular endothelium in anaphylactic shock.

It would seem that the bronchial vascular bed reacts by increased development to those areas of the lung wholly or partially deprived of pulmonary blood or the seat of pathological processes. How far resistance to pulmonary infection is diminished by occlusion of the bronchial arteries by operative interference, as in Karsner's experiments, or by disease, is an interesting speculation and requires investigation.

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RECENT ADVANCES IN THE CHEMISTRY AND BIOLOGICAL ASSAY OF OESTRIN

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In this review no attempt has been made to deal at all fully with the early work that led up to the recognition of the ovary as an organ of internal secretion, or to the older work on the physiological functions of the hormone. This aspect of the problem has already been adequately reviewed elsewhere (Allen and Doisy, 1927; Parkes, 1928, 1929). It has been the aim of the author to review the recent advances in chemical work on the hormone which has culminated in its isolation in a pure crystalline form, and to lay particular stress on some of the older work that made such advances possible.

The recognition many years ago of the ovary as an organ of internal secretion, was naturally followed by numerous attempts to obtain extracts from ovarian tissue that on administration to female animals would be effective in inducing characteristic oestrous changes in the accessory sex organs. For many years such attempts yielded entirely negative or only doubtfully positive results. Active aqueous or saline extracts from ovaries were obtained by Marshall and Jolly (1906) and by Adler (1912). Iscovesco (1912) and Fellner (1913) obtained more clear cut positive results by using extracts of ovarian tissue made with fat solvents. Their work may be fairly considered as the starting point of all subsequent chemical work on the hormone.

Perhaps the greatest difficulty encountered by these early workers was the entire lack of a rapid quantitative method of determining the potency of their extracts. With the introduction of the vaginal smear technique, a roughly quantitative test for oestrus-producing extracts became available and improvements in the methods of extraction and purification of the hormone immediately followed. The availability of more purified extracts then made possible still further improvements in the method of assay. As Parkes (1929) states, "Progress may thus be said to have alternated between advance in the technique of testing and advance in the methods of preparation."

A further bar to progress lay in the lack of a cheap and easily available source of the hormone. The preparation of large amounts of active material from ovarian tissue was an extremely expensive and laborious process on account of the cost of the raw material and of the large volumes of solvents necessary for its extraction. Furthermore, the crude fatty extracts so obtained were difficult to purify and still more difficult to get into a form suitable for administration to test animals without harmful effects. These difficulties were not overcome by the discovery that placentae contained large amounts of the hormone. A cheap and easily available source of the hormone was provided by the discovery of Aschheim and Zondek (1927) that the urine of pregnant women contained large amounts of the active principle which was readily extractable by ether or chloroform. The fundamental importance of this discovery is strikingly shown by the fact that within three years the isolation of the hormone from this source in crystalline form was reported independently from four different laboratories.

NOMENCLATURE. Few substances of physiological interest have been named so frequently as the oestrus-producing hormone. The greater number of the names suggested do not stand a critical examination of their suitability. It appears to the author that on general grounds a suitable name for an endocrine secretion should indicate either the source of the hormone or its function in the body.

The frequently used terms "ovarian hormone" and "female sex-hormone" suggest that only one hormone is elaborated by the ovary. These terms would, of course, be equally suitable for the one or more hormones of the corpus luteum. The same objection applies to the name "feminin" used by Fellner and by Glimm and Wadehn.

The terms "follicular hormone" and "folliculin" are less vague, but they suggest that the hormone is elaborated only in the follicle or that its production is always associated with the follicular phase of the oestrous cycle. The work of Parkes and Brambell (see Parkes, 1929) has clearly shown that the hormone is still produced by the ovary even after complete destruction of the follicular system by x-rays. Furthermore, although the production of the hormone by the ovary is undoubtedly associated with the follicular phase of the cycle in certain animals, this is not invariably the case (e.g., the rabbit and ferret). It is also doubtful if the use of these terms and the term "ovarian hormone" can be justifiably applied to the active substance present in placenta and urine, until the ovarian origin of these principles has been definitely proved.

The name "menformon" suggested by Laqueur and his co-workers

(1925) does not appear to the writer to satisfy the criteria of suitability. It must be pointed out, however, that the name was not applied to the abstract active principle but only to extracts of a certain minimum potency.

Wiesner and his associates use the name "α-hormone." The term is of course entirely meaningless except to those familiar with the work from that particular laboratory.

The term "oestrus-producing hormone," has the advantage of expressing precisely the only known function of the active principle, while it has the added advantage of not making any unjustifiable assumptions about the source of the hormone. The name, however, is cumbersome. The term "oestrin," originally suggested by Parkes and Bellerby (1926) and since widely used in this country and elsewhere, seems to be equally good, however. Veler, Thayer and Doisy (1930) have put forward an objection to this term on the grounds of its use "by phamaceutical houses for extracts containing only the partially purified hormone." It must be pointed out that the name "theelin" suggested by these authors is now also used by a commercial firm for one of its products.

The problem of nomenclature has been recently made still more difficult by the isolation from urine of two chemically related but distinct crystalline substances, both of which are physiologically active. Obviously the name "oestrin" or any of the ones previously suggested cannot be used alone to designate each or both of these substances. Doisy and his co-workers have suggested the names "theelin" and "theelol" for the two compounds (Gk. *thelus*, feminine). Obviously the objections raised to the names "feminin," etc., are here equally applicable. Moreover, although "theelol" suggests the alcoholic nature of the one compound, the name "theelin" affords no clue to the chemical nature of the substance so designated.

The author, however, is unable to see any serious objection to the continued use of the name "oestrin" in certain cases. The term can still be used when it is desired to refer to the active principle in crude extracts or when it is desired to refer to the hormone in an abstract manner in physiological work. The same word, by the addition of suitable suffixes or prefixes, might also be applied to the two crystalline compounds. Butenandt and Hildebrandt (1931) refer to the two compounds as the hormone and the hormone-hydrate to indicate that the second crystalline compound differs from the first by the addition of the elements of water. As a variation of this scheme, the names "oestrin" and "oestrin-hydrate" suggest themselves as possibly suitable names.

The author has made the suggestion (Marrian, 1931) that the names "dioxyoestrin" and "trihydroxyoestrin" might be provisionally adopted since the first named compound contains two oxygen atoms (one hydroxyl and one ketone group), while the second contains three hydroxyl groups. The name ketohydroxyoestrin (Marrian and Haslewood, 1932) is perhaps more suitable than dioxyoestrin, as previously suggested. The objection might be raised that these names suggest that the compounds are derivatives of the active substance *oestrin*, in which case the names could be altered to "ketohydroxyoestrene" and "trihydroxyoestrene," where "oestrene" is used to designate the parent unsaturated hydrocarbon of both compounds. In this review, however, the system previously suggested by the author will be used, except that ketohydroxyoestrin will be substituted for dioxyoestrin.

THE CHEMISTRY OF OESTRIN. *Early methods of preparation.* As has already been pointed out, the first work which had any important bearing on the progress of subsequent work was that of Iscovesco (1912) and Fellner (1913), who showed that fat solvents were superior to aqueous ones for the extraction of oestrin from ovaries or placentae. Hermann (1915) and Hermann and Fraenkel (1915) described a method for the preparation of extracts that formed the basis of all attempts at isolation of the hormone for a number of years. The essential steps in the process were extraction of the tissue with alcohol and ether, precipitation of phosphatides from the extract by addition of acetone with subsequent removal of more inert material by fractionation with cold alcohol. The active principle remained in each case in the soluble fraction. The final product was distilled fractionally at low pressures. The value of this work is that it clearly showed that the hormone was fat-like in its solubilities and that it possessed a high degree of thermostability, since it withstood fractional distillation at fairly high temperatures. This method, with modifications, was subsequently employed by many other workers (c.f. Allen and Doisy, 1923; Dickens, Dodds and Wright, 1925; Parkes and Bellerby, 1926).

The only modification of any real value to subsequent workers was that introduced by Ralls, Jordan and Doisy (1926) who showed that by extraction of active extracts dissolved in 70 per cent ethyl alcohol with petroleum ether, cholesterol and other inert impurities were removed, leaving the hormone in the aqueous alcoholic fraction.

"Water-soluble" preparations. The desirability of obtaining extracts of a degree of purity suitable for clinical administration led to many attempts being made to obtain potent aqueous extracts, in spite of the

earlier work which clearly indicated that the hormone was not water-soluble in the ordinary sense of the term. Zondek and Brahn (1925) were successful in obtaining active aqueous solutions by the extraction of partially purified fat-solvent extracts with dilute acid. This work was followed by similar successful attempts by Laqueur, Hart, de Jongh and Wijsenbeek (1925), Laqueur, Hart and de Jongh (1927), Glimm and Wadehn (1926), Allan, Dickens, Dodds and Howitt (1928), Marrian and Parkes (1929), Thayer, Jordan and Doisy (1928) among others.

As far as it affected the chemistry of the active principle, this phase must be regarded as definitely retrogressive. The tendency was to regard the active principle as a water-soluble substance and its essentially fat-soluble character was in danger of being lost to sight. Attention to the problem of obtaining "water-soluble" extracts also resulted in the main problem of purification of fat-soluble extracts being neglected in many laboratories. Furthermore, in the light of recent findings it is extremely doubtful if the maximum potency of such aqueous solutions would be great enough to cause any effects on injection into women in reasonable volumes. If the human unit is of the same order of magnitude as predicted by Marrian and Parkes (1930), then from data published on the solubility in water of the pure active compounds (Butenandt, 1930b, Marrian, 1930d) it follows that several litres at least of a saturated solution of either ketohydroxy or trihydroxyoestrin would have to be injected to produce any result. On the other hand, the availability of stable active aqueous solutions enabled great improvements to be made in the technique of biological assay.

Resistance to alkaline hydrolysis and the acidic nature of oestrin. It was shown by Doisy, Ralls, Allen and Johnston (1924) that potent extracts could be prepared from liquor folliculi by means of an alkaline hydrolysis and subsequent ether extraction. It was not shown, however, whether or not the hormone was quantitatively concentrated in the unsaponifiable fraction. Similar and more conclusive results showing the high degree of stability towards boiling alkali were obtained by Frank et al. (1926) and Laqueur, Hart and De Jongh (1927). On the other hand, Dickens, Dodds and Wright (1925) reported considerable losses when attempts to concentrate the hormone in the unsaponifiable fraction were made. At this stage of the work, although it was abundantly clear that oestrin was stable to alkalis, it was still uncertain how far ether extraction of the unsaponifiable matter was satisfactory as a means of purification. Evidence was in fact accumulating which suggested that such a procedure resulted in the retention of a considerable

fraction of the potency in the aqueous alkaline soap phase. Thus Allan, Dickens, Dodds and Howitt (1928) showed that on washing an ethereal solution of oestrin with aqueous alkali, some of the active principle passed into the aqueous phase. Glimm and Wadehn (1929) moreover showed that on boiling with alkali, the hormone was converted into an ether-insoluble form.

Funk (1929) showed that the hormone was weakly acidic or phenolic in character and formed salts on treatment with alkalis. These results were confirmed and extended by the writer (1929b). It was shown that urine oestrin, like ovarian and placental oestrin, was quantitatively stable to prolonged boiling with alkalis. The hormone could also be quantitatively extracted from acid or neutral aqueous solutions by ether, but not from alkaline solutions. However, on saturating an aqueous alkaline solution with carbon dioxide, the oestrin could then be quantitatively extracted. It was clear, therefore, that the hormone was a weakly acidic substance.

CRYSTALLINE OESTRIN. The first claim to have isolated the hormone in a chemically pure state was made by S. Fraenkel (1929). Benzene or alcohol extracts of placentae were purified by removal of cholesterol, phosphatides and fatty acids. The "chemically pure hormone" was crystallised from this partially purified extract from ether solution at a low temperature. This substance was stated to have the formula $C_{16}H_{28}O_2$, and to be the lactone of an open chain hydroxy-acid having a double linking between the twelfth and thirteenth carbon atoms. From the few data available to the writer, it is difficult to assess the value of this work, particularly since no mention of the physiological activity of the preparation was made. In the light of more recent work, it is hard to believe that the substance isolated was the pure oestrus-producing principle. It must, however, be borne in mind that the later successful attempts at isolating the hormone have all been made with urine extracts. At the present time it would perhaps be rash to assume that the active principle in pregnancy urine is chemically identical with that in placentae and ovaries.

Wieland, Straub and Dorfmuller (1929) reported the isolation from urine of small amounts of physiologically active crystalline material, after a long and somewhat tedious purification process. Their product softened at 154°, melted at 165° and finally cleared at 210°. As the authors themselves stated, the product was certainly not pure.

Isolation of ketohydroxyoestrin ($C_{18}H_{22}O_2$). The isolation of a highly potent pure crystalline substance was announced almost simultaneously

by Doisy, Veler and Thayer (1929), Butenandt (1929a, b) and by Dingemanse et al. (1930).

The credit for first isolating this crystalline hormone clearly seems to be due to Doisy et al., since the crystals were exhibited by them at the Physiological Congress in Boston in August 1929. Details of the method of preparation of the crystals and their physical and chemical properties were not, however, published until the following year.

In the meantime, Butenandt (1929a, b) described the isolation of an active crystallisate from urine extracts which melted at 240° (uncorr.). The substance had the composition C, 78.31 per cent; H, 8.13 per cent, and from preliminary determinations of the molecular weight and by reason of the fact that it dissolved in alkali only on warming, he suggested that it was a hydroxylactone of the formula $C_{23}H_{28}O_3$ or $C_{24}H_{32}O_3$. Fuller data were shortly afterwards published (Butenandt, 1930b). The melting point was given as 250–251° (corr.) and further analyses clearly pointed to the formula $C_{18}H_{22}O_2$. By the preparation of a mono-oxime and a mono-acetate, it was shown that one oxygen atom was present in a keto group and the other in a hydroxyl group.

A few weeks after Butenandt's preliminary notes, Dingemanse et al. (1930) described the isolation of what was clearly the same substance (C, 78.6 per cent; H, 8.25 per cent, m.p. 240°).

Some months later Veler, Thayer and Doisy (1930) described the preparation of an active compound from urine (m.p. 243–243.5° uncorr.). In a later paper the same authors (Thayer, Veler and Doisy, 1930) gave the corrected melting point as about 249° and characterised the substance as a dihydroxy alcohol of the formula $C_{18}H_{23}O_2$.[1] A year later Thayer, Levin and Doisy (1931, b) rejected this formula and published data confirming Butenandt's findings that the substance was a hydroxyketone of the formula $C_{18}H_{22}O_2$.

The isolation of trihydroxyoestrin ($C_{18}H_{24}O_3$) *and its relation to ketohydroxyoestrin.* Shortly after the first announcements of the isolation of crystalline ketohydroxyoestrin, the author (Marrian, 1930a, b.) isolated from urine a slightly impure active crystallisate which appeared different in many respects to that isolated previously by others. In spite of its slight impurity it showed a higher melting point (256–260° uncorr.), and analyses and molecular weight determinations suggested the formula $C_{18}H_{24}O_3$. In later papers (Marrian, 1930c, d), this substance was obtained in a much purer condition (264–266° uncorr.,

[1] Apparently this is a misprint for $C_{18}H_{24}O_2$ or $C_{18}H_{22}O_2$.

about 281° corr.), and the formula $C_{18}H_{24}O_3$ was confirmed. The preparation of a triacetate showed the presence of three hydroxyl groups.

It was apparent that this active compound was chemically distinct from that isolated by Doisy, Butenandt, and Dingemanse. It was, however, difficult to understand how these authors failed to isolate the triol and why the writer had failed to isolate the lower melting compound. At the time the most probable explanation seemed to be that the treatment with hot alkali in the writer's process of isolation had resulted in the conversion of the keto-hydroxy compound to the trihydroxy substance by the uptake of the elements of water.

The true explanation was clear from important results published soon after by Butenandt (1931a),[2] (Butenandt and Hildebrandt, 1931). By following closely the method of isolation described by the writer, Butenandt was able to isolate the higher melting compound and confirmed its characterisation as a triol of the formula $C_{18}H_{24}O_3$. Butenandt showed, furthermore, that the same substance could also be isolated from one of the discarded fractions in his own method of purification, while ketohydroxyoestrin could be isolated from the mother liquors after isolation of trihydroxyoestrin by the writer's method. It was clear, therefore, that both active crystalline substances co-existed in the original urine. Butenandt then showed that if trihydroxyoestrin was distilled in a high vacuum with potassium bisulphate, the elements of water were removed, the product being ketohydroxyoestrin similar in chemical and physical properties and physiological potency to that isolated directly from urine.

Shortly before the publication of Butenandt's results, Doisy, Thayer, Levin and Curtis (1930) in a preliminary note reported the isolation of a substance (m.p. 273° uncorr.) from one of their discarded fractions from the isolation of ketohydroxyoestrin. This substance was characterized as a triol of the formula $C_{18}H_{24}O_3$. Doisy and Thayer (1931) and Thayer, Levin and Doisy (1931, a) suggested that the writer's previously isolated triol was either a mixture of both active compounds or else an isomeric triol, since there was a difference of some 8° between the published melting points. Clearly, however, these authors were entirely unjustified in basing their criticisms on a difference between *uncorrected* melting points. There is no doubt whatsoever in the writer's mind that the two triols are identical.

Thayer, Levin and Doisy (1931a), in order to explain the isolation

[2] The writer wishes to acknowledge with sincere thanks the kindness of Doctor Butenandt in communicating these results privately to him some time before publication and for sending to him several samples of ketohydroxyoestrin.

of the two active compounds have independently readvanced the explanation previously suggested by the writer and which was shown by Butenandt to be incorrect. They do not appear to have given the attention to Butenandt's important results which should have been accorded to them.

In conclusion it must be pointed out that Butenandt's findings dispose of the possibility that the activity of both compounds is due to an active adsorbed impurity in each, since the conversion of trihydroxyoestrin to the more potent ketohydroxyoestrin was accompanied by an actual increase in total activity.

The urine of pregnant mares as a source of oestrin. Zondek (1930) made the important discovery that the urine of pregnant mares was like human pregnancy urine, a rich source of the hormone.

The question of the identity of the active crystalline substance or substances present has been carefully examined by a number of workers from Laqueur's laboratory (de Jongh et al., 1931; Nieuwenkamp and Kober, 1931; Dingemanse et al., 1931). These workers obtained an active crystalline substance which in chemical and physical properties was identical with their crystalline "menformon" (ketohydroxyoestrin) obtained from the urine of pregnant women.[3]

The physiological potencies of ketohydroxy- and trihydroxyoestrin. As will be pointed out in a later section, the number of variable factors that can influence the vaginal smear test are so many, that it is quite impossible to compare the figures for the potency of either crystalline substance as published from different laboratories. It is, however, interesting to compare the *relative* potencies of the two compounds which have now been published from three different laboratories.

Butenandt (1931a) and Butenandt and Hildebrandt (1931) showed that when tested on mice in a single injection in oily solution, ketohydroxyoestrin was 5.3 times more active than trihydroxyoestrin prepared by the writer's method. Ketohydroxyoestrin, moreover, induced a more prolonged oestrous reaction and there was a longer latent period between the injection and the onset of the oestrous-symptoms than was the case with trihydroxyoestrin. When the tests were made in oily solution with multiple injections the relative potencies of the two compounds fell to about 4 to 1.

Very different results have been recorded by Curtis and Doisy (1931). By injection of the solutions in three doses over 8 hours into rats, it was found that ketohydroxyoestrin was approximately twice as active as trihydroxyoestrin, although the large variations found for different

[3] See Appendix.

preparations of ketohydroxyoestrin leave one in doubt as to what the real potency of the latter substance is. Unlike Butenandt, they found that the reaction due to trihydroxyoestrin is more prolonged than that due to ketohydroxyoestrin. These authors also tested the relative potencies of the two compounds with regard to their power of causing precocious opening of the vaginae of immature rats. The surprising and extremely interesting result was found that trihydroxyoestrin is 6 to 7 times more active than ketohydroxyoestrin in causing this phenomenon.

Experiments by the writer (Marrian, 1931) confirm the figures of Curtis and Doisy. Numerous tests on a large number of different preparations of trihydroxyoestrin gave figures of 7 to 9 million mouse units per gram by the vaginal smear method. Tests on a few samples of ketohydroxyoestrin gave figures approximating to 17 million mouse units per gram.[4] This ratio is in fair agreement with that given by Curtis and Doisy. On the other hand, the writer agrees with Butenandt in finding that ketohydroxyoestrin gives a more delayed and more prolonged reaction than trihydroxyoestrin. It is difficult to see how these results can be reconciled with those of Butenandt which give to ketohydroxyoestrin a much higher relative potency. It must be supposed that the factors which influence the vaginal smear test and which vary in different laboratories according to the technique used, influence the reactions of the test animals towards the two compounds in different degrees. Certainly in the case of the results obtained by the writer and by Butenandt, the differences in the relative potencies must be ascribed to the different testing techniques, since the writer has tested samples of ketohydroxyoestrin prepared by Doctor Butenandt, while the latter has tested one sample of trihydroxyoestrin prepared by the former. The results in both cases agreed well with those obtained on samples prepared in their own respective laboratories.

The question of the relative physiological potencies of the two forms has been made even more difficult by recent work of Butenandt and Hildebrandt (1931) who have obtained from trihydroxyoestrin by frequent recrystallisation a fraction which has only 1/75th of the potency of the original substance. The material so obtained has a somewhat higher melting point than the original substance, but yielded similar combustion figures and had the same optical rotation. Like the origi-

[4] The writer also published results which suggested that ketohydroxyoestrin when freshly prepared existed in a second and much more active form. It has not so far been found possible to repeat these results and the writer now inclines to the view that only one form of ketohydroxyoestrin exists.

nal trihydroxyoestrin, it could be converted into ketohydroxyoestrin by dehydration with potassium bisulphate. It is not at present clear whether this material represents a less active isomer of trihydroxyoestrin, or whether it means that the potency of trihydroxyoestrin is due to the presence of traces of ketohydroxyoestrin. The latter explanation does not appear at all probable to the writer. Ketohydroxyoestrin and trihydroxyoestrin differ so markedly in the durations of the oestrous reactions which they induce, and in the length of the latent period before the reaction commences, that this explanation can only hold, provided one postulates that the inactive substance $C_{18}H_{24}O_3$ actually influences in a qualitative manner the potency of ketohydroxyoestrin. Furthermore, on the basis of the relative potencies for the two compounds found by Curtis and Doisy and the writer, one would have to suppose that in trihydroxyoestrin prepared by the methods described, about 50 per cent was ketohydroxyoestrin. The most probable explanation is that the less active triol isolated by Butenandt and Hildebrandt is indeed an isomer of trihydroxyoestrin.

So far the only physiological effects of the two crystalline compounds studied have been vaginal cornification and induction of precocious oestrus in immature animals (Curtis and Doisy, 1931). Unpublished and as yet incomplete experiments being carried out in collaboration with the writer by Mr. T. F. Dixon indicate that the amounts of ketohydroxyoestrin and of trihydroxyoestrin required to induce full oestrous distention of the uterus in ovariectomised mice are roughly in proportion to the amounts required for vaginal cornification.

Possible physiological rôles of ketohydroxy- and trihydroxyoestrin. Butenandt (1931) has suggested that ketohydroxyoestrin is the actual oestrus producing hormone as it is secreted by the ovary, and that prior to excretion in the urine it is converted to trihydroxyoestrin in order to increase its water-solubility. On this theory the latter compound must be regarded merely as an excretion product with no definite physiological rôle in the body.

The recent work of Curtis and Doisy (1931) already mentioned which has shown trihydroxyoestrin to be much more active in inducing precocious oestrus than ketohydroxyoestrin is of particular interest and importance since it suggests a possible physiological rôle for the former substance.

It does not appear to the writer that at the present stage one is justified in suggesting that either compound is the oestrus producing hormone of the ovary itself. The position at present seems to be merely that two active crystallisates have been isolated from the urine of preg-

nancy, which differ in relative physiological potencies according to what criterion of activity is taken. There seems to be every reason to suppose that the ultimate source of these substances is the ovary, but there is no reason to suppose that the ovary secretes them in the forms in which they are found in urine. Only isolation of the active crystalline substance from ovarian tissue can settle this point.

Chemical and physical properties of ketohydroxyoestrin and its derivatives. Optical rotation. The following data have been published on the optical rotation of ketohydroxyoestrin:

AUTHORS	SOLVENT	CONCENTRATION	WAVE LENGTH	TEMPERATURE	$[\alpha]$
		grams per 100 cc.			
Butenandt (1930.b)	Chloroform	0.935	5890	18°	+156°
Thayer, Levin and Doisy (1931b)	95 per cent ethyl alcohol	0.107	5890	Not given	+166°
	95 per cent ethyl alcohol	0.580	5890	Not given	+156°
	95 per cent ethyl alcohol	0.073	5890	Not given	+157°
Marrian and Haslewood (1932)	Ethyl alcohol	0.2675	5461	19°	+188°

Absorption spectrum. Butenandt (1930b) has shown that ketohydroxyoestrin has a well-defined absorption band in the ultra-violet at 283–285 $\mu\mu$.

Solubility. Ketohydroxyoestrin is fairly easily soluble in alcohol, acetone, benzene and chloroform. It is less soluble in ether and still less soluble in petroleum ether. According to the figures given by Butenandt (1930b) its solubility in water is about 0.0015 per cent.

Ketohydroxyoestrin esters. On treatment with acetic anhydride in pyridine solution a crystalline monoacetate is formed which may be recrystallised from dilute alcohol; m.p. 125.3° (uncorr.) (Thayer, Levin and Doisy, 1931b), 126° (uncorr.) (Butenandt, 1930b). Butenandt has found that the physiological potency of the acetate is almost the same as that of ketohydroxyoestrin. Thayer, Levin and Doisy have shown that hydrolysis of the acetate leads to the recovery of ketohydroxyoestrin identical in all respects with the original material.

Butenandt (1930b) has also prepared a bromacetate, m.p. 133–147° and a benzoate, m.p. 211–212° (uncorr).

Compounds of ketohydroxyoestrin by virtue of the carbonyl group. Treatment of ketohydroxyoestrin in alcoholic solution with hydroxylamine acetate yields a crystalline monoöxime which melts with decomposition at 229–230° (uncorr.) (Butenandt 1930b; Thayer, Levin, and Doisy 1931b).

Butenandt and Hildebrandt (1931) have also prepared a semicarbazone, m.p. 245–246° with evolution of gas and decomposition.

Ketohydroxyoestrin monomethyl ether. Thayer, Levin and Doisy (1931b) have shown that on treatment of ketohydroxyoestrin in alkaline solution with dimethyl sulphate a monomethyl ether is formed, m.p. 165° (uncorr.).

Marrian and Haslewood (1932) found that a sample of ketohydroxyoestrin methyl ether (m.p. 164–167°), prepared in the same way had $[\alpha]_{5461}^{20} = +171°$.

Unsaturation of ketohydroxyoestrin.[5] Butenandt (1930b) has shown that on catalytic hydrogenation ketohydroxyoestrin is converted into a reduction product $C_{18}H_{30}O$, m.p. 104° (uncorr.). This would seem to suggest that three double bonds are present in the molecule.

Thayer, Levin and Doisy (1931b) determined the iodine value of ketohydroxyoestrin by an unpublished method of Jordan and Ralls. Their figures indicate the presence of one double bond only.

Marrian and Haslewood (unpublished work) have determined the iodine value of the monomethyl ether of ketohydroxyoestrin and have obtained almost theoretical figures for one double bond.

Chemical and physical properties of trihydroxyoestrin and its derivatives: Optical rotation. The following data have been published on the optical rotation of trihydroxyoestrin.

AUTHORS	SOLVENT	CONCENTRATION	WAVE LENGTH	TEMPERATURE	$[\alpha]$
		grams per 100 cc.			
Marrian (1930.d)	Pyridine	2.70	5461	Not given	+38.6°
Marrian and Haslewood (1932)	Pyridine	2.58	5461	22°	+42.9°
	Pyridine	3.35	5461	23°	+40.8°
	Ethyl alcohol	0.432	5461	21°	+78.3°
	Ethyl alcohol	0.261	5461	22°	+73.6°
Butenandt and Hildebrandt (1932)	Pyridine	Not given		Not given	+34.4°
	Pyridine	Not given	5461	Not given	+38.5°
Thayer, Levin and Doisy (1931a)	95 per cent ethyl alcohol	0.4016	5890	27°	+61°

[5] See Appendix.

Solubility. Trihydroxyoestrin is fairly soluble in alcohol and acetone. It is less soluble in ether and ethyl acetate, and still less soluble in petroleum ether. In water, at about 20°C., the solubility is of the order of 0.003 per cent.

Trihydroxyoestrin acetate. On acetylation of trihydroxyoestrin with acetic anhydride, a triacetate is formed, m.p. 120–122° (uncorr.) (Marrian, 1930d), 126° (uncorr.) (Thayer, Levin and Doisy, 1931a). Both Marrian (1930d) and Thayer, Levin and Doisy (1931a) have shown that hydrolysis of the triacetate yields the original trihydroxyoestrin.

Trihydroxyoestrin mono-methyl ether. Treatment of trihydroxyoestrin with dimethylsulphate in alkaline solution yields a monomethyl ether, m.p. 154.8° (Thayer, Levin and Doisy, 1931a), 162.5–164° (uncorr.) (Marrian and Haslewood, 1932). Butenandt and Hildebrandt (1931) by methylation with diazomethane have prepared a monomethyl ether, m.p. 169°. The following data on the optical rotation of trihydroxyoestrin methyl ether have been published:

Butenandt and Hildebrandt (1931), $[\alpha]_D$ in pyridine = +34.7°
Marrian and Haslewood (1932), $[\alpha]_{5461}$ in pyridine = +29.9°
Marrian and Haslewood (1932), $[\alpha]_{5461}$ in alcohol = +65.3°

Unsaturation of trihydroxyoestrin.[6] Thayer, Levin and Doisy (1931a) determined the iodine value of trihydroxyoestrin and obtained a value agreeing closely with that required for one double bond. Marrian and Haslewood (unpublished work), have obtained figures closely agreeing with this finding in determinations of the iodine value of trihydroxyoestrin monomethyl ether.

It is clear, therefore, that the degree of unsaturation of trihydroxyoestrin is the same as that of ketohydroxyoestrin.

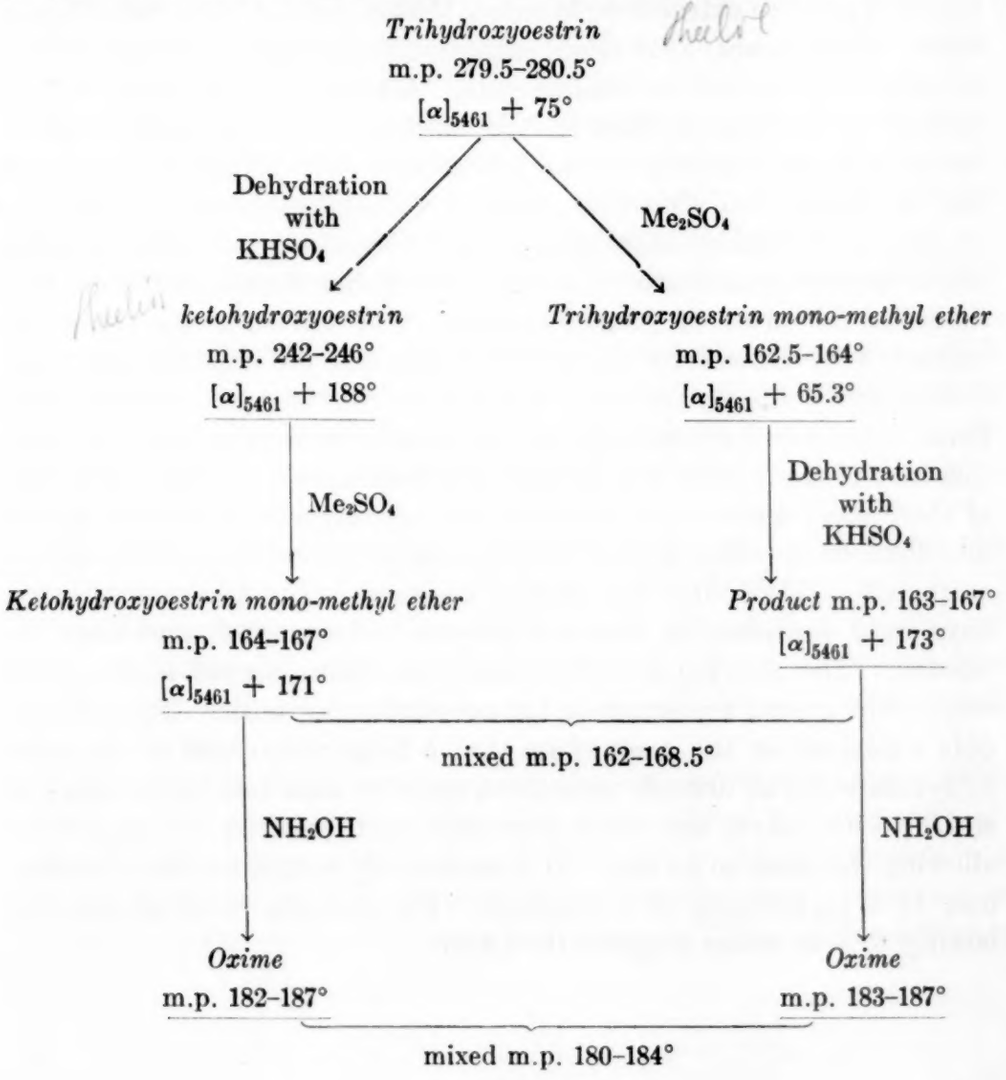
The mechanism of the conversion of trihydroxyoestrin to ketohydroxyoestrin. Butenandt (1931) suggested that the acidity of ketohydroxyoestrin is due to the enolisation of the carbonyl group. Thayer, Levin and Doisy (1931a), on the other hand, since they have prepared a monomethyl ether which is insoluble in alkali, have suggested that the acidic properties are due to the hydroxyl group. If Butenandt's suggestion is correct, it must be supposed that in the conversion of trihydroxyoestrin to ketohydroxyoestrin the phenolic hydroxyl of the former is involved. If the views of Thayer, Levin and Doisy are correct, then water must split out from the two non-acidic hydroxyls of trihydroxyoestrin to give ketohydroxyoestrin, leaving the phenolic hydroxyl intact.

More recently Butenandt (1931b) has agreed with Thayer, Levin and Doisy, in believing the acidity of ketohydroxyoestrin to be due to the hydroxyl group, since he has found that while derivatives of the hydroxyl

[6] See Appendix.

group are not soluble in alkali, although retaining their ketonic properties, those in which the ketone group is involved are still soluble in alkali.

More clear-cut experimental evidence bearing on this point has been recently advanced by Marrian and Haslewood (1932). They showed that trihydroxyoestrin monomethyl ether on dehydration yielded a product that could be oximated, and which was identical in rotation, melting point and crystalline form with ketohydroxyoestrin monomethyl ether. Furthermore, the latter product yielded an oxime that was identical with the oxime of the dehydration product of trihydroxyoestrin monomethyl ether. These results can only be explained by supposing that on conversion of trihydroxyoestrin to ketohydroxyoestrin water is eliminated from the two non-acidic hydroxyls of the former, and that therefore the acidity of the latter is also due to a phenolic hydroxyl group. This work is summarised in the accompanying table.



METHODS OF ISOLATION OF THE ACTIVE CRYSTALLINE COMPOUNDS. The first step in the isolation of the crystalline compounds from the urine of pregnancy is the extraction of the material by some suitable fat solvent which is immiscible with water and in which the active principles are readily soluble. The writer has invariably used ether and it has unfailingly yielded satisfactory results. It is in many ways, however, an unsuitable solvent, particularly for large scale work. The losses due to destruction of the hormone by peroxides may be considerable unless freshly redistilled ether is used. There is some risk from fire, and the recovery of the solvent, particularly in warm weather, makes the process uneconomical. Doisy and co-workers have in turn employed ether (Veler and Doisy, 1928), olive oil (Doisy, Veler and Thayer, 1930) and more recently chloroform and butyl alcohol (Veler, Thayer and Doisy, 1930).

The yield of active material can be greatly increased if the urine is acidified prior to extraction (Marrian, 1930aa; Doisy, Veler and Thayer, 1930). This is not, as it might appear, due merely to the active compounds being present as alkaline salts. Absolutely fresh urine which is neutral in reaction or slightly acid, still gives an increased yield of oestrin if acidified strongly before extraction. The pK of trihydroxyoestrin is about 10.5 (Marrian, 1930d), ketohydroxyoestrin is an even weaker acid: clearly, therefore, no salt formation can occur in urine under normal conditions. Zondek (1930) has found that the hormone in the urine of pregnant mares is in a form which cannot be extracted by chloroform directly. Butenandt (1931a) has suggested that oestrin is mainly present in urine as an ester of a polyhydroxy acid. Some unpublished experiments by the writer confirm this idea. A large quantity of fresh urine was divided into four equal portions. The first of these was acidified and extracted immediately with ether, the second and third were boiled in acid and alkaline solutions respectively before extraction. The fourth was allowed to stand in the laboratory for ten days until considerable bacterial growth had occurred, and then extracted. The activity of each extract was then assayed in the usual way. The results are shown in the accompanying table. They can be only explained on the assumption that a large proportion of the total active material in urine is present as an ester with some acid which is stable to hot alkali, but which is readily hydrolysed by hot acid or by allowing the urine to go bad. It is tentatively suggested that the ester may be a glycuronate or a sulphate. The stability to alkali and the lability to acid rather suggests the latter.

	YIELD OF ACTIVITY FROM ETHER EXTRACT IN M.U. PER LITRE OF URINE
Fresh urine, acidified	1,250
Boiled with acid	5,600
Boiled with alkali	2,070
Allowed to decompose	>12,500

In order to obtain maximum yields of oestrin from urine, it is necessary to hydrolyse this ester completely in some way. It is clearly impracticable to allow large volumes of urine to decompose, although the experiments described above show that this is a highly efficient method. It is probably sufficient to allow the urine to stand in the cold for some days after acidification (Doisy, Veler and Thayer, 1930), to heat the urine with acid for a short time, or to evaporate the urine to a small bulk after acidification.

The next stage in the process consists of separating from the crude extract the active phenolic fraction. Not only is there a large quantity of acidic substance in the extract (e.g., benzoic acid), but also a large neutral unsaponifiable fraction containing cholesterol, pregnandiol (Marrian, 1929a; Dingemanse et al., 1930; Butenandt, 1930c), a paraffin hydrocarbon and other substances of unknown composition.

The acid fraction may be conveniently removed by washing an ether solution with aqueous sodium carbonate or bicarbonate, or by an alkaline saponification followed by ether extraction after saturation with carbon dioxide. Some of the acidic constituents appear to be present as esters hydrolysable only by acids, since more inert material can be removed by either of the above processes if the material is given a preliminary acid hydrolysis.

The phenolic fraction may be separated from the remainder of the material by extraction of an ethereal solution with aqueous alkali.

The final stage consists of separating from the active phenolic fraction, fractions containing ketohydroxyoestrin and trihydroxyoestrin respectively, and the isolating and purification of the active compounds from these fractions.

The method of Doisy and co-workers (Veler, Thayer and Doisy, 1930; Doisy and Thayer, 1931). The urine was acidified to congo red, allowed to stand for a week and then extracted with butyl alcohol in a continuous extractor. The butyl alcoholic extract was evaporated to dryness and the residue extracted with benzene. The residue left after

evaporation of the benzene extract was extracted with *N/2* sodium hydroxide after the addition of a small volume of butyl alcohol; the alkali was partially neutralised and after chilling the clear solution was poured off from the tar that separated.

The aqueous alkaline solution was extracted with ether, the ether evaporated and the dry residue steam distilled to get rid of volatile substances. The residue not steam distillable was extracted several times with hot alkali and after filtration through a Jena glass filter the alkaline extract was thoroughly extracted with ether. The ether extract after washing with *N/10* sodium bicarbonate, *N/10* hydrochloric acid and finally water, was evaporated and the residue extracted repeatedly with cold *N/4* sodium hydroxide. The alkaline extract was extracted with ether, washed as before and evaporated to dryness. The product was a stiff yellow oil in which crystals appeared upon standing.

From this product crude crystals of ketohydroxyoestrin were separated by crystallisation from aqueous alcohol. The crystals were purified by recrystallisation from butyl alcohol-petroleum ether mixtures, and from aqueous alcohol.

This process was designed for the isolation of ketohydroxyoestrin before the existence of the second active compound was suspected. Large losses of active material, however, occurred in the stages where the hormone was extracted from aqueous alkaline solution by ether. Doisy and Thayer (1931) were therefore led to examine these extracted aqueous alkaline fractions—particularly the penultimate one in which most of the loss occurred. After boiling off the dissolved ether and acidifying, crude crystalline trihydroxyoestrin was precipitated. This was decolourised with charcoal and recrystallised from aqueous alcohol.

This process is, in the opinion of the writer, rather unnecessarily long and tedious. The authors have, however, clearly shown that ketohydroxyoestrin, although acidic in character, can be extracted from alkaline solution by ether leaving the trihydroxyoestrin in the aqueous alkaline phase. Presumably this is due to dissociation of the sodium salt of ketohydroxyoestrin which is thus clearly a less strongly acidic substance than trihydroxyoestrin. This process provides an excellent method for the separation of the two active substances from one another.

The method of Butenandt (Butenandt, 1930a; 1931a). The starting material was a crude urine extract prepared by Schering Kahlbaum. A. G., Berlin. This was dissolved in 50 per cent aqueous methyl alcohol and extracted with high boiling petroleum ether. Benzoic acid and cholesterol were removed by the petroleum ether, leaving the hormone

in the aqueous alcoholic phase. The latter after dilution with water was ether extracted. The residue from the evaporated ether extract was dissolved in 70 per cent aqueous ethyl alcohol and extracted with benzene. The benzene extract was washed with 60 per cent methyl alcohol. It was shown that the benzene extracted the ketohydroxyoestrin, while the trihydroxyoestrin was retained in the aqueous alcoholic phase.

The residue from the evaporated benzene extract was boiled with alcoholic hydrochloric acid, diluted with water and ether extracted. Washing of this ether extract with aqueous sodium carbonate removed much inactive acidic material. The washed ether solution was extracted with aqueous alkali to extract the active material, leaving pregnandiol in the ethereal solution. The alkaline, extract after acidification was extracted with ether, the ether washed and evaporated to dryness. The product was an oil from which crystalline ketohydroxyoestrin was obtained by a slow fractional sublimation in a high vacuum. The retort was first heated for 12 hours at 100–115° at 0.20 −0.03 mm., when 20–25 per cent of the oil distilled over. The temperature was then raised for about 60 hours from 120°–220°, when crystals of the hormone sublimed in the neck of the retort. The greater part of the hormone distilled at about 150°. The crystals were washed with cold ether to remove traces of oily material and then crystallised from a mixture of ethyl acetate and petroleum ether.

Trihydroxyoestrin was isolated from the aqueous alcoholic phase after the removal of the ketohydroxyoestrin by extraction with benzene.

The method of Marrian. The method described here is considerably modified from the methods described previously (Marrian, 1930b, d). Certain of the processes originally suggested by Doisy and co-workers and by Butenandt have been introduced with advantage. As far as the preparation of trihydroxyoestrin is concerned, the method is essentially the same as that used by Marrian and Haslewood (1932).

The urine was collected without any preservation and allowed to stand in a warm room for about 10 days. It was then extracted with ether after acidification with sulphuric acid. The residue after evaporation of the washed ether extract was heated for 2 hours with 5 per cent aqueous potassium hydroxide. The alkaline solution after saturation with carbon dioxide was thoroughly extracted with ether. The residue from the evaporated ether extract was heated with alcoholic hydrochloric acid, diluted with water and the mixture ether extracted. The ether extract was washed first with aqueous sodium carbonate to remove inert

acidic constituents set free by the acid hydrolysis and then with aqueous 5 per cent potassium hydroxide, to extract the active phenolic fraction. The pregnandiol remained in the ethereal phase. The active material was extracted from the aqueous alkali by ether after saturation with carbon dioxide. The ether solution after washing with water was dissolved in 60 per cent aqueous ethyl alcohol and extracted with benzene. The benzene was washed with aqueous methyl alcohol.

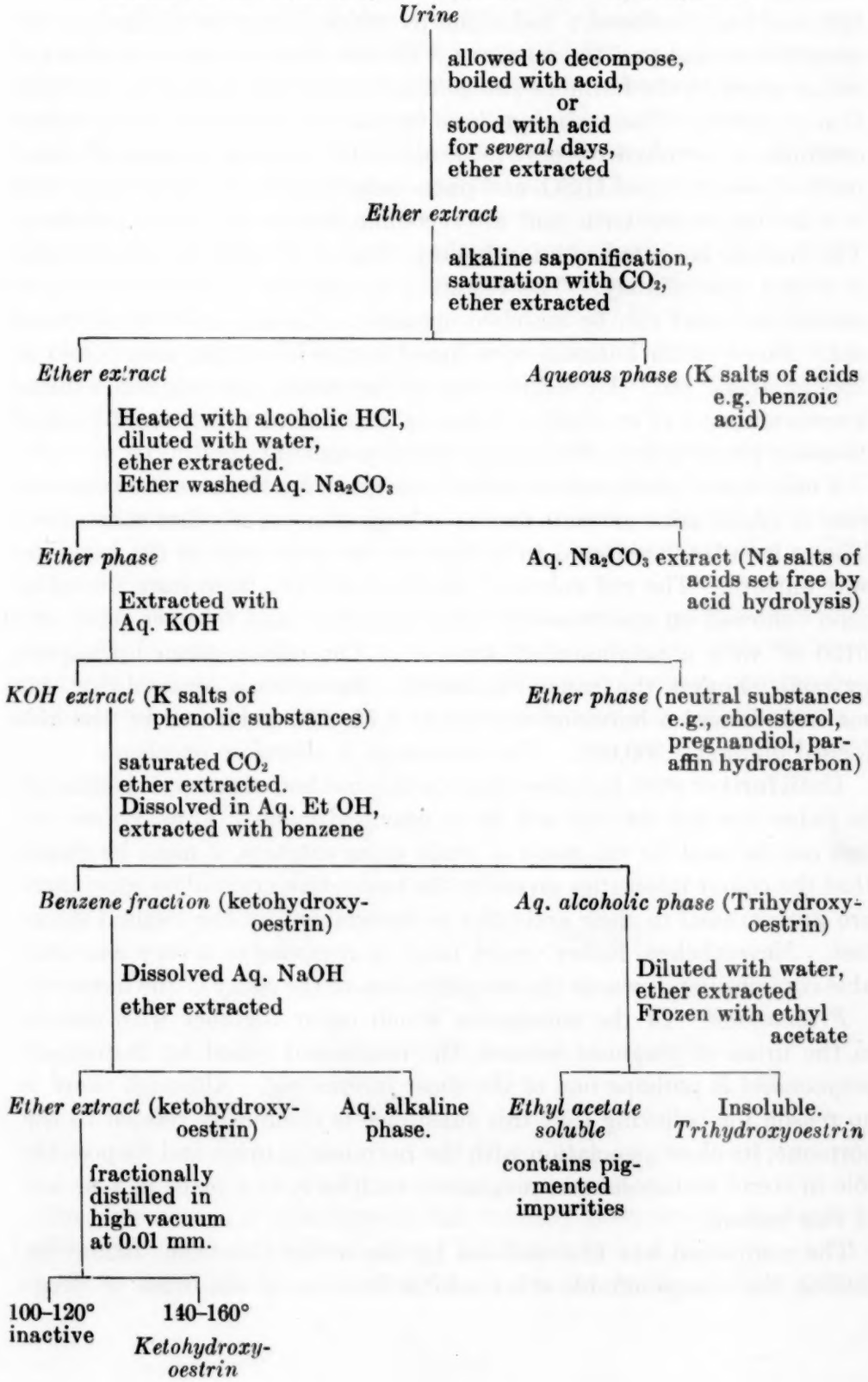
a. *Trihydroxyoestrin.* The combined aqueous alcoholic fractions after partial evaporation were diluted with water and extracted with ether. Crude trihydroxyoestrin separated from the residue from the evaporation of the ether on chilling with a small volume of ethyl acetate. The crude crystals were decolourised by boiling with charcoal in alcoholic solution. A further treatment of the crystals with a small volume of ice-cold ethyl acetate removed most of the remaining pigmented impurities. Purification was effected by dissolving in alkali, filtering and precipitating with carbon dioxide, finally recrystallising from aqueous ethyl alcohol.

b. *Ketohydroxyoestrin.* The benzene solution containing the ketohydroxyoestrin was evaporated to dryness and treated with a large volume of *N/100* aqueous sodium hydroxide. The active substance was extracted from the mixture by repeated extraction with ether, leaving a large proportion of inactive impurities in the aqueous alkali. The residue obtained by evaporation of the washed ether extract was distilled in the manner described by Butenandt and yielded crystals of ketohydroxyoestrin. The whole process is shown diagrammatically in the accompanying figure on page 205.

THE COLORIMETRIC DETERMINATION OF OESTRIN. Apart from the colour reactions given by the two crystalline substances by virtue of their phenolic hydroxyls and their aromatic nuclei (cf. Marrian 1930 c,d), the only other clearly defined coloured reaction that has been observed is the reddish yellow colour given on warming with concentrated H_2SO_4.

Wieland, Straub and Dorfmuller (1929) tested their impure crystallisates by both the Salkowski and the Liebermann-Burchardt sterol tests. In the former test the H_2SO_4 layer was coloured yellow, while in the latter it was a reddish-yellow with a strong green fluorescence. The writer (Marrian, 1930b) observed the same phenomenon with the Liebermann-Burchardt test. In a later paper (Marrian 1930d) it was shown that pure trihydroxyoestrin gave the same colour reaction merely on warming with concentrated H_2SO_4.

Scheme for the isolation of keto-hydroxy- and trihydroxyoestrin



Recently, Kober (1931) has made a careful examination of this reaction and has developed a technique by which it may be applied to the quantitative assay of the hormone. He has shown, moreover, that the colour given by the hormone can be considerably intensified by the addition of phenol. The technique is as follows: To about 5 γ of crystalline menformon (ketohydroxyoestrin) is added 0.2 cc. of a mixture of equal parts of concentrated H_2SO_4 and phenolsulphonic acid. This is warmed in a boiling water bath and after cooling, 0.2 cc. of water is added. The mixture is again heated to boiling, diluted with 0.6 cc. of water and is cooled immediately. The resulting mixture is a clear red without fluorescence and can be matched against a standard solution of cresol red. Five γ of the hormone were found to give about the same colour as 25γ of cresol red. By calibration of the cresol red solution against known amounts of crystalline hormone, a standard which could be used to assay preparations of unknown potency was obtained.

Under these conditions no colour was given by the inactive constituents of crude urine extracts and by a large number of other substances. The only substance found to behave in the same way as the hormone was anthrol. The red coloured reaction solution from pure "menformon" showed on spectroscopic examination a band between 4150 and 5150 A° with a maximum at 4880 A°. The colour given by impure extracts showed the same maximum. An extract assayed by this method showed a hormone content of 3,750,000 units and by the biological method 3,300,000. The agreement is therefore excellent.

Until further work has been done on this method of assay, it is difficult to judge how far the test will be of practical value. Thus, before the test can be used for the assay of crude urine extracts, it must be shown that the colour intensities given by the two active crystalline substances are proportional to their activities as determined by the vaginal smear test. Nevertheless, Kober's work must be regarded as a very considerable contribution towards the simplification of the assay of the hormone.

Pregnandiol. Of the substances which occur together with oestrin in the urine of pregnant women, the compound called by Butenandt *pregnandiol* is perhaps one of the most interesting. Although there is no reason for believing that this substance is chemically related to the hormone, its close association with the hormone in urine and its possible rôle in sterol metabolism in pregnancy entitles it to a place in a review of this nature.

The compound was first isolated by the writer (Marrian, 1929a) by chilling the unsaponifiable ether-soluble fraction of the urine of preg-

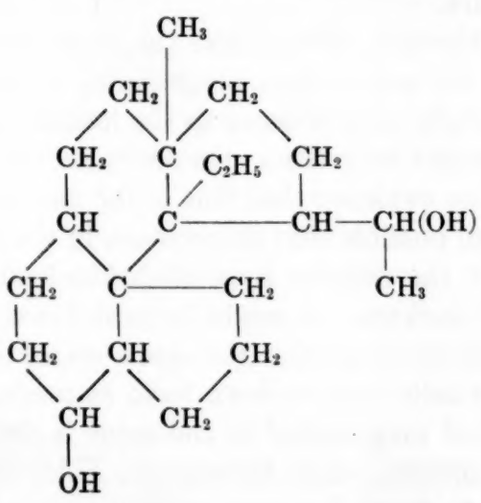
nancy, with a small quantity of ether or acetone, when white crystals of substance remained undissolved (m.p. 233–235° uncorr.). Yields up to 2 mgm. per litre were obtained. The compound proved to be saturated, to have no oestrus-producing potency, and was characterised by an extremely low solubility in most of the usual organic solvents.

Analyses and molecular weight determinations suggested the molecular formula $C_{19}H_{32}O_2$ or $C_{20}H_{34}O_2$. By the preparation of a diacetate it was shown that both oxygen atoms were present as hydroxyl groups.

Shortly afterwards the isolation of what was clearly the same compound was briefly reported by Dingemanse et al. (1930). A more complete examination of the substance was made by Butenandt (1930c), who confirmed most of the writer's general observations, but showed that the compound was more probably represented by the formula $C_{21}H_{36}O_2$.

By the oxidation of pregnandiol with cold chromic acid Butenandt obtained a diketone $C_{21}H_{32}O_2$ (m.p. 123°), showing that both hydroxyl groups were secondary. The ketone gave a positive iodoform reaction, indicating the presence of a $-CO-CH_3$ group, and therefore of a $-CH(OH)-CH_3$ group in pregnandiol itself.

By more vigorous oxidation with hot chromic acid, a ketodicarboxylic acid $C_{21}H_{32}O_5$ was obtained (m.p. 270° decomp.). On the basis of these experiments Butenandt suggested that pregnandiol was a neutral degradation product of the sterols with the following structural formula.



The correctness of this formula has recently been proved by further brilliant researches of Butenandt, Hildebrandt and Brucher (1931) who have provided clear evidence for its relation to the cholane group.

Pregnandiol was oxidized to the diketone *pregnandione*, and the latter then reduced by the Clemmensen method to the parent saturated hydrocarbon *pregnane* $C_{21}H_{36}$, m.p. 83.5°.

The ethyl ester of cholanic acid (I) on treatment with methyl magnesium iodide yielded dimethyl-norcholyl-carbinol (II). The latter on oxidation with chromic acid yielded norcholanic acid (III), which was then converted into its ethyl ester and treated with phenyl magnesium bromide giving bisnorcholyldiphenyl carbinol (IV). This substance was converted into α-bisnorcholanic acid (V) by oxidation with chromic acid. The methyl ester of (V) was treated with phenyl magnesium bromide, yielding a carbinol (VI). The latter was heated with acetic acid and then distilled in a high vacuum at 200–240°. Water was split out quantitatively from the carbinol yielding a hydrocarbon $C_{34}H_{44}$ (VII). This hydrocarbon on treatment with ozone yielded benzophenone and aetio-cholyl-methyl ketone (VIII). The latter on reduction yielded a hydrocarbon $C_{21}H_{36}$ which was shown to be identical with pregnane prepared from pregnandiol.

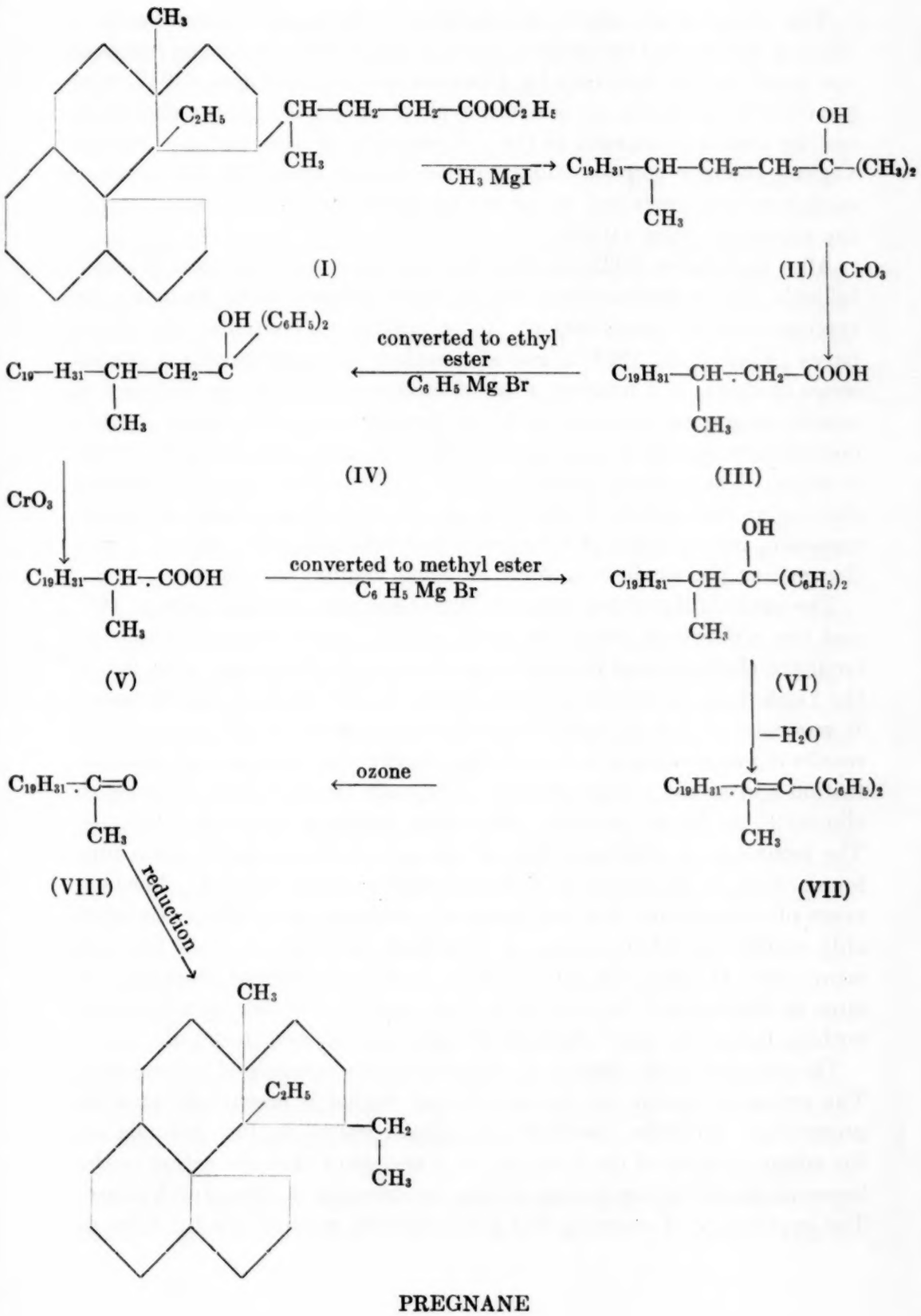
The question of the relation of pregnandiol to the sterols is thus satisfactorily settled, and on the assumption that the Wieland-Windaus structures for cholesterol and the bile acids are correct, its structure may also be regarded as proved.[7]

It would appear that the preparations originally obtained by the writer and which led him to suggest the formulae $C_{19}H_{32}O_2$ or $C_{20}H_{34}O_2$, were slightly impure.

It was shown (Marrian, 1929a) that the urine of males and of non-pregnant females did not contain pregnandiol in detectable amounts. Its apparent specificity to pregnancy in the human naturally led to the suspicion that it might be related chemically to the oestrus-producing hormone. So far no evidence that this is the case has been forthcoming. It would seem possible that its presence in the urine in pregnancy may be a result of the definite hypercholesterolemia which has been observed by many workers. It might be postulated that the pregnant human being unable to metabolise the excess sterol by the usual paths, excretes it in a partially broken-down form as pregnandiol.

The appearance of pregnandiol in the urine is clearly not specific to pregnancy in all species, since Butenandt, Hildebrandt and Bucher (1931) were unable to detect it in extracts of the urine of pregnant mares. It would be interesting to know whether mares exhibit a high blood-cholesterol during pregnancy.

[7] See Appendix.


CH3
C2H5
CH—CH2—CH2—COOC2H5
CH3
CH3 MgI
C19H31—CH—CH2—CH2—C—(CH3)2
OH
CH3
(I)
(II)
CrO3
OH (C6H5)2
C19—H31—CH—CH2—C
CH3
converted to ethyl ester
C6 H5 Mg Br
C19H31—CH—CH2—COOH
CH3
(IV)
(III)
CrO3
C19H31—CH—COOH
CH3
converted to methyl ester
C6 H5 Mg Br
OH
C19H31—CH—C—(C6H5)2
CH3
(V)
(VI)
—H2O
C19H31—C=O
CH3
ozone
C19H31—C=C—(C6H5)2
CH3
(VIII)
reduction
(VII)
CH3
C2H5
CH2
CH3

PREGNANE

THE BIOLOGICAL ASSAY OF OESTRIN. *The vaginal smear method.* Most of the present methods of assay of the oestrus-producing hormone are based on the discovery by Stockard and Papanicolaou (1917) that the different stages in the oestrous cycle of the guinea pig are characterised by clear-cut changes in the cell contents of the vagina. Similar vaginal changes corresponding to the various stages of the oestrous cycle were demonstrated in the rat by Long and Evans (1922), and in the mouse by Allen (1922).

Allen and Doisy (1923) showed that the injection of extracts of liquor folliculi into ovariectomised rats or mice induced in 40–48 hours the typical oestrous "cornification" of the vaginal cell contents. In a later paper (Allen et al., 1924) it was shown that by administering a graded series of doses to a number of ovariectomised animals, the potency of oestrus-producing extracts could be roughly assayed. Such roughly quantitative methods were used during the next few years by many workers. The general practice seems to have been to inject graded doses of an oil solution of the hormone, or of an oil—sodium carbonate emulsion, into a series of ovariectomised rats and mice, and to define the unit as the smallest amount inducing vaginal cornification.

The availability of less impure "water-soluble" extracts (see p. 188) and the widespread desire for more strictly quantitative methods to facilitate chemical and physiological work on the hormone, soon led to the publication of several critical papers on the assay of the hormone. It was realised that in order to obtain even approximately quantitative results it was necessary to standardize rigidly the conditions of administration and to use a large number of animals for each test, in order to eliminate, as far as possible, differences between individual animals. The technique of administration of extracts to test animals has a profound effect on the intensity of the oestrous reaction induced. For purposes of convenience this technique of administration differs considerably in different laboratories. It is difficult therefore to make any fair comparison between the quantitative results of different workers. It must be emphasised that the terms "rat unit" or "mouse unit" convey nothing unless the exact method of assay is described in detail.

The influence of the number of injections on the intensity of the response. The action of oestrin on the uterus and vagina is essentially growth-promoting. In order, therefore, to induce oestrus in test animals by the administration of the hormone, it is necessary that the action of the hormone should be continuous during the duration of this growth phase. The importance of ensuring this was originally pointed out by Allen et

al. (1924), who recommended administering the hormone by three subcutaneous injections at intervals of 4–8 hours in corn-oil solution to facilitate its slow absorption and to maintain a high concentration in the blood over the requisite period.

In the case of "water-soluble" preparations, the effect of multiple injections is extremely marked. Laqueur and his colleagues (c.f. Laqueur and de Jongh, 1929) showed that the effect of a given dose of "menformon" in aqueous solutions in six injections over a period of 48 hours was very considerably stronger than the effect of the same total amount administered in one injection. These findings were subsequently confirmed by many other workers (c.f. Allan, Dickens, Dodds and Howitt, 1928; Marrian and Parkes, 1929). The principle of adopting some measure to ensure delay in the rate of absorption is now accepted by most workers. If aqueous solutions are being tested, the best method is to administer multiple injections. In the case of oil solutions subdivision of the dose is not so necessary. Satisfactory results have been obtained by workers who have administered single injections of oil solutions to their test animals (Coward and Burn, 1927; Butenandt and von Ziegner, 1930).

The view has been expressed that for comparative physiological tests it is not necessary to ensure delayed absorption, since the *comparative* potencies of different solutions will be the same whether administered in one or many doses. This, however, would only be true when the rate of absorption of the different solutions was exactly the same. This point is best illustrated by an example.

Suppose that A and B are respectively aqueous and oily solutions containing the same concentrations of the pure hormone and nothing else. If the solutions are tested by a single injection method, solution B will apparently be considerably more potent on account of the delay in absorption of the hormone into the blood stream by the oil. If the solutions are tested by a four injection method such as that of Marrian and Parkes (1929), the higher activity of B will still be apparent but will not be so marked. If the six injection method of Laqueur is used, the apparent potencies of the solutions will be still more nearly the same. The slowing up of the rate of absorption artificially by dividing the dose thus tends to cover up errors which might result from the different rates of absorption of the solutions themselves.

The number and frequency of the vaginal smears. The necessity of recording a sufficient number of vaginal smears from each animal so that no positive reaction is missed, was stressed by Laqueur and de

Jongh (1929). The author is completely in accord with these views, and it is his conviction that lack of attention to this point is one of the most frequent sources of error in the biological assay of the hormone. In his experience the smears should be commenced at least 8 hours before any positive reaction is expected and continued at intervals of not more than 8 hours until the smears are entirely negative. In certain cases it may even be advisable to smear more frequently than this.

The view has been expressed to the writer that this precaution is not necessary in the case of tests on large groups of animals, since if infrequent smears are taken at certain fixed hours (e.g., twice a day at 9 a.m. and 5 p.m.), the number of animals showing a positive response will still bear a relation to the amount of the hormone injected. Such a method can only give accurate comparative results if the active preparations administered all induce their positive responses at the same period after injection. It would thus be impossible to compare the potencies of ketohydroxyoestrin and trihydroxyoestrin in this way, since the oestrous response induced by the former is several hours later than that induced by the latter. Infrequent smears at fixed times would therefore be likely to give one or other compound a relatively higher activity than it actually possessed. In the same way, comparison of the relative vaginal units of different species of animal are probably only accurate if all positive reactions are recorded, since there are no grounds for believing that the duration of the period between injection and induced oestrus is the same for all species.

The oestrous response following the injection of a single dose of an aqueous solution of the hormone is extremely brief, only lasting in some cases an hour or so. The slowing of the rate of absorption by the use of oil solutions or by splitting the dose causes a marked prolongation of the duration of the vaginal response. It may be laid down as a general rule, therefore, that the smaller the number of injections, the more frequent must be the recording of vaginal smears to obtain reliable results. The writer has found that it is quite possible to determine accurately the potency of pure aqueous solutions of the hormone by the single injection method if vaginal smears are recorded often enough.

Weight of animal. The possible influence of the body weight upon the amount of oestrin required to induce a positive response in test animals has been studied by many workers. Working on the rat, Bugbee and Simond (1926) found that older and heavier animals required more hormone to produce a positive reaction, and they suggested applying a correction for the variation in body weight. Also working on the

rat, d'Amour and Gustavson (1930) showed that groups of heavier animals required consistently greater doses than groups of smaller animals. The ratio of the units for the two groups was less than the ratios of their average body weights and they concluded "that there is not simple relationship between body weight and the rat unit." The variation of the unit with weight was in their experiments insufficient to justify a body weight correction.

Laqueur and de Jongh (1929) with the mouse could find no evidence that the weight of the animal was of any significance. Marrian and Parkes (1929) also working with the mouse, found slight evidence that there was some relationship between body weight and dose; their figures were, however, not statistically significant and they concluded that in routine tests a weight correction was unnecessary.

From purely theoretical considerations it must be supposed that there is some relationship between the size of animal and the dose of oestrin required to induce oestrus. The variations in body weight between different adult animals from the same colony are generally so small, however, that this factor may be safely neglected, particularly if the group method of testing is used (see p. 215).

The relation between the rat and mouse unit. Since rats and mice are the most commonly used animals for assaying oestrin, the question of the relative magnitude of the rat and mouse unit of the hormone is of some importance. Laqueur and de Jongh (1929) give the ratio of the rat unit to the mouse unit as 4 to 1. They do not, however, mention the average weight of the rats used, thus it is impossible to see if this ratio has any relationship to the relative body weights of the two species.

An attempt was made by Professor Dodds and Doctor Dickens in conjunction with the writer (unpublished work) to clear up this point. Tests on a standard aqueous solution of oestrin were carried out on rats by the former and on mice by the writer. As far as possible the technique of smearing and of evaluation of the smears was standardised in the two laboratories. Tests both by the six dose method of Laqueur and by the four dose method of Marrian and Parkes were carried out. By both methods it was found that the rat unit equalled approximately 10 mouse units. While it is realised that it is unsafe to compare results obtained in two different laboratories, it is nevertheless somewhat significant that the average body weight of the rats used by Professor Dodds was approximately 10 times that of the mice used by the writer.

Coward and Burn (1927) came to the surprising conclusion that there was no difference between the rat and the mouse unit. Laqueur and

de Jongh (1929) suggest that their findings may be the result of not employing the divided dose method. Recently Becker et al. (1931) have repeated this work of Coward and Burn, adopting, like them, a technique of single injections of the hormone dissolved in oil. They find as Coward and Burn did, that the rat unit is the same as the mouse unit.

The writer is perhaps hardly entitled to cast doubt upon these results, since he has not repeated the experiments by the technique adopted by these authors. On the other hand, in collaboration with Doctor Parkes, he has had considerable experience in injecting oestrin into still larger animals, and it has been invariably found that the amount required to produce definite oestrous symptoms is approximately proportional to the body weight of the animal. Furthermore, it is impossible to dismiss the figures of Laqueur and de Jongh, since these workers have probably had more experience of the assay of the hormone than any others.

Possibly the explanation of Coward and Burn's results advanced by the latter workers is correct. An alternative explanation may lie in the vaginal smears being recorded at too infrequent intervals. Neither Coward and Burn, nor Becker et al., however, described their smearing technique in detail.

Evaluation of vaginal smears. There is considerable difference of opinion among different workers as to what constitutes a positive vaginal reaction. Some workers are content with the disappearance of leucocytes, others insist on the complete cornification of all cells. Since it is highly unlikely that it will ever be possible, or even advisable to standardise the technique of assay, the point is not of much importance, except in so far as a lax criterion of what is a positive reaction may lead to errors in assay. Thus, it is not advisable to accept a pure "nucleated" pro-oestrus smear as positive, since there may be a tendency to confuse this type with the normal operative dioestrus smear.

Individual variations among test animals. The earlier methods of assay of oestrin consisted of injecting graded doses of the extract into a series of ovariectomised animals, defining as the unit the smallest amount necessary to cause vaginal cornification. Coward and Burn (1927) pointed out that individual variations in sensitivity among different animals might lead to an error of as much as 1000 per cent when such a method was used. They proposed that a method based on the principles laid down by Trevan for the biological assay of insulin and digitalin should be employed. The method consisted of injecting each of a series of groups of at least twenty animals with different amounts

of a standard preparation of oestrin. The percentage of each group showing a positive response was then plotted against the amount of oestrin administered. The amount corresponding to a 50 per cent response was defined as the unit. In order to assay the potency of any extract, all that was necessary was to inject the same amount into each of 20 mice and after determining the percentage positive response, to calculate the number of units or fraction of a unit received by each animal by reference to the previously constructed curve. This method has since been adopted by several workers, notably Allan, Dickens, Dodds and Howitt (1928), Marrian and Parkes (1929), and d'Amour and Gustavson (1930), with considerable success. Marrian and Parkes (1929) found that by such a method, oestrin could be assayed with an error of not more than ± 10 per cent.

Although many other workers have not adopted this method, it is now generally recognised that unless a large number of animals are used for each test, the results are unlikely to be accurate. Both Kahnt and Doisy (1928), and Laqueur and de Jongh (1929) insist on using a large number of animals for each test and define as the unit that amount required to cause a 75 per cent response.

With regard to the Coward and Burn method of assay, it must be emphasised that the shape of the initial standardisation curve probably varies with the degree of homozygosity of the colony of animals used, and therefore separate curves must be constructed by each worker. To what extent the shape of the curve depends upon the number of injections made and upon the degree of purity of the extracts used is not entirely certain. The unpublished experiments previously referred to, which were made by the writer in conjunction with Professor Dodds and Doctor Dickens indicated, however, that the shape of the curves was the same when Wistar rats of inbred albino mice were used, or when a 6 injection or a 4 injection technique was employed. Further unpublished experiments by the writer also have shown that similar curves are obtained with the 4-injection technique with crude urine extracts or with pure trihydroxy-or ketohydroxyoestrin. The writer has also constructed a series of curves by using 1-injection technique with crystalline ketohydroxyoestrin in aqueous solution. In this case, however, the curve differed markedly in shape from the previously mentioned ones. It was not found possible to induce more than 60 per cent of each group of mice to show a positive response even when very large doses were given.

Effect of previous injections on the sensitivity of test animals. A possible source of error in any method of assay lies in the fact shown by several

workers that after a large dose of oestrin, test animals are for some time abnormally sensitive to subsequent doses. Kahnt and Doisy (1928) observed that the degree of genital atrophy that occurs after ovariectomy influenced the amount of oestrin subsequently required to produce a positive smear. They therefore recommended the priming of each animal with an injection of oestrin one week before the tests are to be made. In the course of routine tests they suggested that the animals should be used once a week and that all animals showing a negative reaction in one test should be primed with 1.5 units the following week before being used for further tests. d'Amour and Gustavson (1930) showed that groups of animals which have received a large dose of oestrin always showed a higher percentage response the following week to a given amount of the hormone, than animals which had received previously a smaller dose. They correctly have pointed out that priming the test animals results in a smaller unit. A similar phenomenon has frequently been observed to occur by the writer. He has found, however, that to obtain regular results it is not necessary to prime the animals, provided that they are injected with not more than 1.5 units on alternate weeks (Marrian, 1929b). Coward and Burn (1927) also recommended using the test animals not more frequently than once a fortnight. As a further precaution the writer has found it advisable to mix thoroughly the individual animals in the various testing groups of twenty before each fortnightly test.

VAGINAL SMEAR METHOD OF ASSAY—SUMMARY

For the guidance of those who wish to develop their own methods of assay, the following principles may be laid down:

a. Some method of securing delayed absorption (divided doses or oil solutions) should be employed to ensure that differences in apparent potency of extracts is not due to differences in their degree of purity which will affect their rates of absorption.

b. Vaginal smears should be recorded frequently so that none or only a few of the total positive reactions be missed.

c. A sufficient number of animals should be used to guard against errors due to individual variations in sensitivity of the animals used.

A number of critical studies of the methods employed for the biological assay of oestrin have been published. Among the most important of these are Coward and Burn (1927), Laqueur and de Jongh (1928), Kahnt and Doisy (1928), Marrian and Parkes (1929) and d'Amour and Gustavson (1930).

The writer wishes to take this opportunity of expressing his sincere thanks to the Medical Research Council, who have so generously financed his own work, to the various hospital authorities who have provided facilities for the collection of urine, and to Schering-Kahlbaum, A. G., Berlin who have kindly supplied several samples of urine extract.

APPENDIX ADDED IN PROOF

Since the foregoing was written, several important papers have been published on the chemistry of oestrin.

Adam et al., and Bernal (Journ. Soc. Chem. Ind. 1932, li, 259) have shown by measurements on monomolecular films and by crystallographic measurements that the hormone has a fused ring structure, possibly being a phenanthrene derivative. Later work by the former (in press) has proved that the two alcoholic hydroxyls in trihydroxyoestrin or the ketonic group in ketohydroxyoestrin must be at the opposite end of the molecule to the phenolic hydroxyl.

Girard et al. (C. rend. l'Acad. Sci., 1932, cxciv, 909 and 1020) have shown that the urine of pregnant mares contains besides ketohydroxyoestrin, two active crystalline substances $C_{18}H_{20}O_2$, which differ in optical rotation from the former.

Butenandt et al. (Zeitschr. f. physiol. Chem. 1932, ccviii, 129 and 149) claim that ketohydroxyoestrin exists in two isomeric modifications. The question of the unsaturation of the crystallisates is examined. It is concluded that an "enolic" double bond is present in the molecule in addition to the three benzene ring double linkages and that the acidic nature of the one hydroxyl group is due to this bond. The writer, on the basis of unpublished experiments, believes that the evidence for a double bond other than those in the benzene nucleus is not entirely conclusive. Butenandt et al. conclude that the hormone has a phenanthrene skeleton.

Rosenheim and King (Journ. Soc. Chem. Ind. 1932, li, 464) have suggested that sterols have a chrysene skeleton. If this suggestion proves to be correct, not only will Butenandt's pregnandiol formula have to be revised, but the relationship between sterols and pregnandiol on the one hand and the hormone on the other hand becomes a possibility.

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THE CHEMISTRY OF THE CARDIAC GLUCOSIDES

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The cardiac drugs are substances which have been grouped together because of their possession of a common characteristic action on the heart. The present review will be restricted to an outline of their chemistry and it is not the intention to give more than passing reference to the pharmacodynamic or biological aspects of our knowledge concerning them. Such a treatment has already been so admirably presented in the classical monograph of Arthur Cushny. With the exception of the toad poisons, these substances are all of vegetable origin. Although the number of plants from which they have been obtained is somewhat limited, such plants are of wide geographical distribution. Many species are to be found in tropical and subtropical regions and because of their poisonous properties were the source of many arrow and ordeal poisons. The distribution in the vegetable kingdom is also rather wide. The majority of plants in which they have been found appear to be embraced by the natural order *Apocynaceae*, but families scarcely related to this, such as the *Scrophulariaceae*, the *Liliaceae*, and the *Ranunculaceae*, contain species which yield such poisons.

There has been a gradual accumulation of data from the attempts to study the chemistry of the active constituents of these plants, but most of this until recently has been of a preliminary character. The fact that most often not one but a number of substances make up the poisonous principle has been partly responsible for this. The relatively small amounts which as a rule occur in the plants have added to the difficulties. Finally, such substances are of a very complex structural character. In many cases their isolation in crystalline form proved difficult, and with few exceptions where crystalline substances could be secured they were later shown to be mixtures. But the recent introduction of special methods of procedure in individual cases has permitted definite progress in their study to be made. In the limited space of this review we must restrict ourselves to an occasional brief indication of these methods.

With but few exceptions, the active substances are glucosides and

consist entirely of carbon, hydrogen and oxygen. The exceptions are erythrophleine, the nitrogenous principle of *Erythrophloeum guineense*, and the toad venoms. The glucosides on treatment with acids are hydrolyzed with the production of the component sugars and the non-carbohydrate constituents, the aglucones or "genins" as they have been termed. In a number of instances the sugars have proved of interest because of the occurrence in them of certain unusual structural features which will be discussed in passing.

For the proper presentation of the chemistry of these substances it will be necessary to describe first the individual glucosides as they have been isolated from the various plants. Following this, the molecular structure of the aglucones, as far as this has been determined, and their interrelationships will be discussed.

Since the pioneer observations of Withering on the therapeutic powers of the foxglove in the treatment of dropsy and the correlation by subsequent observers of these effects with a characteristic and specific action on the heart, a host of reports of studies with this drug has accumulated, which far exceed the studies on any other cardiac drug. However, our knowledge of the real chemistry of the active principles of *Digitalis purpurea* is a comparatively recent development. This has been a very slow process, greatly hampered by the fact that the biologically active fraction of the drug consists of an undetermined number of substances, each one of which is in itself a complex molecule. In addition, there are present equally complex saponins and their derivatives which exhibit no cardiotonic activity and which are extracted along with the active glucosides. Because of their effects on the solubility relationships, they have greatly complicated the problem of the isolation of chemical individuals from the mixture.

Both the seeds and especially the leaves of the plant have been used for the production of a number of therapeutic preparations. The active glucosides which preponderate in the former, while closely related, have been shown to be substances quite distinct from those which occur in the latter. The glucosides of the seeds can be entirely removed by extraction with water (1). Such an extract contains a complex mixture of active and inactive glucosides. Only one of the active glucosides has been recognized as a chemical individual, the digitalin of Schmiedeberg (2), who also discovered its glucosidic character. Kiliani (3), who used the commercial *Digitalinum pur. pulv. germanic.* (of Merck) as his starting material, later obtained digitalin in practically pure form, and confirmed its chemical individuality. He gave it the name *digi-*

talinum verum, and at the same time developed a practicable method for its separation from other substances. Although he succeeded in obtaining it in crystalline form, it usually deposits in the form of amorphous granules. The glucoside when pure is very sparingly soluble in water, but its occurrence in the original aqueous extract is caused by the presence of large amounts of saponins, especially digitonin and gitonin and other extractives which readily form colloidal solutions. There are unquestionably other active glucosides in the extract, especially the so-called digitalein of Schmiedeberg (4), a mixture of substances which has been provisionally characterized by its solubility in water. However, nothing is known of their chemistry.

Kiliani has shown that *digitalinum verum*, for which he adopted a formula $C_{35}H_{56}O_{14}$, yields on hydrolysis an aglucone, digitaligenin, and two component sugars, digitalose and glucose. The more recent work of Windaus and co-workers (5) has definitely shown the necessity of revision of the formula of the glucoside. This is most likely $C_{36}H_{56}O_{14}$, and that of its hydrolytic product, digitaligenin, is definitely $C_{23}H_{30}O_3$. They have also proved that digitaligenin does not occur preformed in the glucoside, but that it is a dehydration product of the original aglucone, $C_{23}H_{34}O_5$, which is very probably gitoxigenin. The comparatively severe conditions required for the hydrolytic cleavage of the glucoside cause the simultaneous removal of 2 mols of water from this aglucone with the formation of an unsaturated dianhydrogitoxigenin or digitaligenin, $C_{23}H_{30}O_3$. This is shown in the following scheme.

$$C_{36}H_{56}O_{14} + 2H_2O = C_{23}H_{34}O_5 + C_6H_{12}O_6 + C_7H_{14}O_5$$
$$C_{23}H_{34}O_5 - 2H_2O = C_{23}H_{30}O_3$$

Digitalose has been found by Kiliani (6) to be a methyl ether of a methyl pentose. We shall later note two other methyl ether sugars, cymarose and sarmentose, which have been found associated only with the group of cardiac glucosides. These are the only methyl ether sugars which have been isolated from natural sources. In *digitalinum verum* it is probable that digitalose is the sugar directly joined to the aglucone and that glucose is attached to one of the remaining hydroxyl groups of the digitalose molecule. This is suggested by the fact that crystalline oleandrin, which will be described further on, has been shown by Windaus and Westphal (24) to be a glucoside of an aglucone probably identical with gitoxigenin and of a sugar possibly digitalose. The addition of a glucose molecule to oleandrin could possibly give *digitalinum verum*.

A more complicated picture is presented by the glucoside mixture contained in the digitalis leaves. This is a subject which has been clouded by much confusion and has caused considerable difference of opinion. However, certain facts have emerged which are now definite. There are two crystalline glucosides present in the extracts which have now been repeatedly isolated in pure form—digitoxin and gitoxin. A third glucoside has also been described by Cloetta, termed *Gitalinum cristallisatum.* Contrary to the conditions encountered with digitalis seeds, roughly two-thirds of the active glucosides can be extracted with water (1). The remainder can be extracted only with alcohol, preferably of 50 per cent. But separation of the individual glucosides in comparatively pure form entails very large losses.

Digitoxin has been found mainly in the alcoholic extract of the leaves after their preliminary exhaustion with water. It is obtained by careful fractionation of the material which is extracted from this solution with ether. It unquestionably occurs also in the aqueous extract. Digitoxin is the main constituent of the original "digitaline crystallisée" of Nativelle who apparently was the first to have isolated it. It was subsequently studied by Schmiedeberg (7) who originated the name digitoxin. Among others who investigated the substance were principally Kiliani (8), then Krafft (9), and more recently Cloetta (10), who was perhaps the first to have obtained it in really homogeneous form, and finally Windaus and co-workers (11). It is characterized by its solubility in chloroform and alcohol and by its very sparing solubility in water. Kiliani was the first to recognize its glucosidic character. On hydrolysis with acid he showed that it is very easily cleaved to digitoxigenin and digitoxose, an α-desoxysugar, $C_6H_{12}O_4$. The correct formulation of digitoxin has only recently been shown by Windaus (12) to be $C_{41}H_{64}O_{13}$ and consists of digitoxigenin, $C_{23}H_{34}O_4$, joined in glucosidic union with 3 mols of digitoxose. It is hydrolyzed by acid in accordance with the following scheme:

$$C_{41}H_{64}O_{13} + 3H_2O = C_{23}H_{34}O_4 + 3C_6H_{12}O_4$$

In this glucoside only one of the digitoxose molecules is directly joined to the aglucone.

Krafft (13) assigned chemical individuality to a water-soluble glucoside which could be extracted from the aqueous extract of the leaves with chloroform and which he called "gitalin." This substance, for which great lability was claimed, deposited a sparingly soluble, so-called dehydration product, "anhydrogitalin," when allowed to stand in

solution or especially when its aqueous solution was heated. In a series of articles Kiliani (14) showed that "gitalin" is a complex mixture of substances. More recent work of Windaus and Schwarte (15) and Cloetta (16) has shown that the so-called "anhydrogitalin" is but an impure form of a glucoside which occurs already in the digitalis leaves and which is not a transformation product. It is characterized by its insolubility in alcohol and in chloroform. It is also the main constituent of an insoluble by-product which has been obtained by E. Merck, Darmstadt, by Hoffmann, La Roche and by other firms in the course of the preparation of crystalline digitoxin. This product appears as the purification of the crude drug proceeds and is collected by virtue of its sparing solubility in chloroform.

The glucoside has been called gitoxin by Windaus and Schwarte (*Digitalinum cristallisatum* by Cloetta). In more recent work Windaus, Westphal and Stein (17) have derived the correct formula, $C_{41}H_{64}O_{14}$, for it. It consists of an aglucone gitoxigenin, $C_{23}H_{34}O_5$, joined to 3 mols of digitoxose which are readily obtained on hydrolysis with dilute acid.

$$C_{41}H_{64}O_{14} + 3H_2O = C_{23}H_{34}O_5 + 3C_6H_{12}O_4$$

On treatment with alcoholic hydrochloric acid the aglucone readily loses 2 mols of water with the formation of a dianhydro derivative which is identical with digitaligenin, the product of the hydrolysis of *digitalinum verum*. As previously mentioned, gitoxigenin is therefore to be regarded as the aglucone of the latter but is converted to digitaligenin by dehydration under the severe conditions necessary for the hydrolysis of this glucoside.

The easy hydrolysis of digitoxin and gitoxin is definitely attributable to the fact that they are glucosides of an α-desoxysugar, digitoxose. We shall note later similarly labile glucosides of two other α-desoxysugars, cymarose and sarmentose. In only one other connection has an α-desoxysugar been obtained from natural sources. This is the α-desoxyribose which Levene has recently demonstrated to be the sugar constituent of thymus nucleic acid.

A third crystalline glucoside of the leaves has been described by Cloetta, to which he has given the name *Gitalinum cristallisatum* (16). This glucoside on hydrolysis was observed to yield 2 mols of digitoxose and 1 mol of a new aglucone, gitaligenin. Gitaligenin was shown to be closely related to gitoxigenin because of its apparent ready conversion into derivatives of the latter on acylation and dehydration. However,

the formulations derived by Cloetta for the glucoside of $C_{17}H_{28}O_6$ and for the aglucone of $C_{11}H_{18}O_3$ are untenable, as discussed by Windaus (18) and Jacobs and Gustus (19). The titration of the lactone group of the glucoside and of the aglucone demonstrated the latter to be of the same order of magnitude as the other genins. Windaus concludes from his own and Cloetta's data that gitaligenin is a hydrate of gitoxigenin of the formula $C_{23}H_{36}O_6$, and that the glucoside has a formula in the neighborhood of $C_{35}H_{56}O_{12} \cdot 1/2\ H_2O$. This substance however, is in need of reinvestigation.

There is finally a question as to the identity of another crystalline glucoside which Kiliani (20) at one time designated as β-digitoxin but later regarded as identical with digitoxin. However, he had obtained on hydrolysis with stronger acid an aglucone derivative, a so-called β-anhydrodigitoxigenin of melting point 223°, which is apparently a different substance from ordinary anhydrodigitoxigenin for which melting points of 183° and 193° have been observed.

Digitalis lanata—another species of digitalis—has been observed by a number of investigators to exceed considerably in activity that of *Digitalis purpurea.* This fact can be attributed to a higher content of cardiotonic glucosides or to the presence of more potent glucosides or to both. Each of these possibilities has been discussed. The chemistry of the glucosides contained in the leaves of this plant has recently received attention. Smith (21) and Mannich, Mohs and Mauss (22) have shown it to be a complex mixture of substances, some of which are identical and others closely related to those found in *Digitalis purpurea.* One of these described by Smith is a new glucoside, digoxin, which is isomeric with gitoxin, $C_{41}H_{64}O_{14}$. On hydrolysis it yields digoxigenin, $C_{23}H_{34}O_5$, which is isomeric with gitoxigenin, and also 3 mols of digitoxose. In addition, Smith obtained gitoxin. The results of the German workers are more involved and differ from those of Smith. They have reported four glucosides of which the principal one is *lanadigin* with the formula $C_{41}H_{66}O_{17}$. On hydrolysis an aglucone, $C_{23}H_{34}O_5$, lanadigigenin, was obtained, which proved to be identical with Smith's digoxigenin. Digitoxose was found in the sugar fraction and also apparently a disaccharide, $C_{12}H_{22}O_9$, which was more resistant to acids. From these results lanadigin can be interpreted as a glucoside of digoxigenin with 1 mol of digitoxose and 1 mol of a hexosedigitoxose disaccharide perhaps conjugated as a triose.

A second glucoside of unusually complex character and provisionally called Lanata-glucoside II yielded on hydrolysis two genins—digoxi-

genin and digitoxigenin—and as sugars, digitoxose and the [illegible]ve disaccharide of lanadigins. This glucoside maintained the appearance of chemical individuality in all attempts at fractional recrystallization. A third glucoside is apparently *digitalinum verum*. Finally, the fourth glucoside appears to be of unusual character, for which a formula of either $C_{29}H_{48}O_{11}$ or $C_{42}H_{66}O_{16}$ was considered. On hydrolysis it gave glucose and a genin with the formula of either $C_{23}H_{32}O_3$ or $C_{30}H_{42}O_4$.

Nerium oleander, of the family *Apocynaceae*, is a plant the poisonous properties of which were already known in antiquity. The leaves contain unquestionably a mixture of glucosides the chemistry of which was given a provisional and superficial study by Schmiedeberg (23). This has recently assumed a definite form. The laboratories of C. F. Boehringer and Söhne, Mannheim-Waldhof have prepared a crystalline *oleandrin* from the leaves of this plant, although in very poor yield. Oleandrin has been shown by Windaus and Westphal (24) to be a glucoside, $C_{31}H_{48}O_9$, which on hydrolysis yields digitaligenin and a sugar which is probably digitalose. We have already described its possible close relationship with *digitalinum verum* of *Digitalis purpurea* seeds, which has an additional glucose molecule in its structure.

On turning to the family of *Apocynaceae* the most frequent occurrence of plants containing cardiac poisons is found. Most important of these are a number of *Strophanthus* species which grow in tropical Africa. The seeds and in some cases the bark of these plants have been the source of arrow poisons. In the earliest work with these poisons some uncertainty prevailed as to the botanical identity of the seeds from which the strophanthins had been obtained. This was due to the incompleteness of botanical data and to the fact that the *Strophanthus* species are quite numerous. As order was created from this confusion, certain *Strophanthus* species were made official, viz., *S. kombé*, *S. hispidus* and later *S. gratus*. In order to distinguish the strophanthins occurring in the various species, Thoms (25) proposed as a prefix the first letter of the species in which it occurs—as k-strophanthin, h-strophanthin, g-strophanthin, etc. Recently the chemistry of a number of these substances has been largely developed at the Rockefeller Institute.

The classical studies of Fraser (26) and the later work of Feist (27) on the Kombé arrow poison have demonstrated the glucosidic character of the material from which an aglucone, strophanthidin, can be obtained on hydrolysis. The uncertainty of the earlier work, caused by confusion as to the identity of the *Strophanthus* species which were used, has been clarified by the critical review of Heffter and Sachs (28). The latter

reported on the chemistry of the strophanthins isolated from both *kombé* and *hispidus* seeds. The identical aglucone, strophanthidin, was obtained from both. Kombé seeds were found, however, to yield two strophanthidin glucosides—a crystalline k-strophanthin which is sparingly soluble in water, and an amorphous k-strophanthin which is easily soluble in water.

The recent work of Jacobs and Hoffmann has shown that none of these strophanthins is an individual substance. As a matter of fact, the seeds of *Strophanthus kombé* contain a mixture of active glucosides. Practically all of these are derivatives of the one aglucone, strophanthidin, for which Jacobs and Heidelberger (29) derived the correct formula $C_{23}H_{32}O_6$. Jacobs and Hoffmann (30) have separated the so-called crystalline k-strophanthin into its component glucosides by making use of their differential solubilities. Those which were isolated as individuals are cymarin, a chloroform-soluble substance, and K-strophanthin-β, which is practically insoluble in this solvent. Cymarin itself was first isolated by Taub and Fickewirth of Farbenfabriken vorm. Friedr. Bayer & Co. (31) from a number of species of the genus *Apocynum* (*cannabinum*, *androsaemifolium* and *venetum*). It was shown by Windaus and Hermanns (32) to possess the formula $C_{30}H_{44}O_9$ and to be a glucoside of strophanthidin and cymarose, a sugar of the formula $C_7H_{14}O_4$. The latter is the methyl ether of an α-desoxysugar which is either identical or isomeric with digitoxose. Here again, as in the case of the digitalis glucosides, the glucosidic union between the aglucone and the desoxysugar is very readily broken on hydrolysis with acids with the formation of strophanthidin and cymarose.

K-strophanthin-β, $C_{36}H_{54}O_{14}$, is a more complex glucoside which on hydrolysis yields strophanthidin and a bioside, $C_{13}H_{24}O_9$, of cymarose and glucose. This biose in turn cannot be further hydrolyzed into the component sugars since the conditions necessary for disruption of the union between the glucose and cymarose molecules are so vigorous as to cause decomposition of the sugar. The nature of the biose has been indirectly determined by enzymatic cleavage of K-strophanthin-β (33). An enzyme, strophanthobiase, is present in strophanthus seeds, which possesses the property of hydrolyzing the union between the two monoses without affecting the union between the aglucone and cymarose. The result is the production of 1 mol of cymarin and 1 mol of glucose.

The greater proportion of the strophanthin in kombé seeds is the so-called amorphous k-strophanthin. This is also a mixture of water-soluble glucosides which can be readily hydrolyzed to cymarin and

glucose by means of the enzyme. The analysis of the purified amorphous strophanthin has indicated that the preponderating substance is a strophanthidin trioside consisting of a chain of two glucose molecules attached to the cymarose molecule. In addition there are doubtless more complex strophanthins containing a chain of three or more glucose molecules attached to cymarin. In kombé strophanthin, therefore, strophanthidin is directly joined in glucosidic union only with cymarose and not with glucose. Jacobs and Hoffmann (34) have likewise shown that cymarin is also a component of the active principle of *S. hispidus*, which has likewise proved to be a mixture of strophanthidin glucosides. Here again cymarin occurs free and combined with glucose. The preponderating material is a water-soluble, amorphous mixture of the more complex glucosides from which no other crystalline individual has been isolated. By enzymatic treatment, however, it has been possible to obtain cymarin from this mixture, but the hydrolysis to cymarin proceeds with much greater difficulty than in the case of kombé strophanthin. There are, therefore, certain structural factors, unknown at present, which contribute to the greater stability of the hispidus strophanthin. In hispidus strophanthin the strophanthidin molecule is again probably in direct glucosidic union only with cymarose. Although closely related as cymarin-glucose combinations, kombé and hispidus strophanthins are definitely mixtures of different substances, with the exception of free cymarin. As described below strophanthidin glucosides are also present in the seeds of *S. eminii*. Strophanthidin glucosides doubtless occur in still other species of *Strophanthus*. Authoritative information, however, is not at the moment available on this point.

A very interesting observation which the writer has made in connection with the study of the strophanthins of *S. kombé* is the occurrence in the seeds of a relatively very small proportion of a glucoside, allocymarin (35), which is isomeric with cymarin. Allocymarin yields allostrophanthidin and cymarose on hydrolysis. The isomerism is confined to the aglucone molecule. Allocymarin owes its origin to an enzymatic isomerization which occurs in the strophanthidin molecule while still in the glucosidic combination. On self-digestion of a suspension of ground and defatted seeds, while hydrolysis of the glucose molecules occurs, there is an independent although simultaneous rearrangement of strophanthidin to allostrophanthidin. The preponderating substance which results if digestion is allowed to continue is allocymarin. It is of interest that the typical cardiac action of the molecule is almost if not entirely destroyed by the change.

Another important species of *Strophanthus* is *S. gratus.* The seeds of this plant, which were the source of an arrow poison—the inée of the Pahouins—, have been shown by Arnaud (36) to be rich in a beautifully crystalline glucoside, ouabain, as well as an amorphous fraction the chemistry of which has not been explained. Ouabain was first discovered by Arnaud in the roots and bark of the ouabaio tree, a species of *Acocanthera* used also as an arrow poison by the Somalis of East Africa. Because of the homogeneity and excellent physical properties of this glucoside, it has been widely used as a standard for the assay of digitalis and strophanthus extracts. Arnaud originally derived a formula for ouabain of $C_{30}H_{46}O_{12}$ and showed that it is a rhamnose glucoside of a hypothetical aglucone, $C_{24}H_{36}O_8$. Jacobs and Bigelow (37) have recently revised the formulae of ouabain and its aglucone to $C_{29}H_{44}O_{12}$ and $C_{23}H_{34}O_8$ respectively. Ouabain in its stability towards the hydrolytic action of acids differs greatly from the above strophanthins which are glucosides of α-desoxysugars. Such severe conditions as regards the strength of acid and the duration of the reaction are, therefore, required that the aglucone is decomposed with the formation of obscure resinous dehydration products. Therefore, the aglucone of ouabain has never been obtained. In the recent work of Jacobs and Bigelow crystalline derivatives of this aglucone have been secured. Instead of the usual hydrolytic methods it was necessary to employ a special procedure.

A species of *Strophanthus* has been investigated by Jacobs and Heidelberger (38) which was considered to be *S. sarmentosus.* The seeds of this plant contain a mixture of glucosides which have yielded to the same method already employed in the study of kombé strophanthin. One of the glucosides, sarmentocymarin, can be separated from the more complex substances by extraction with chloroform. It is a crystalline substance which is very sparingly soluble in water and possesses the formula $C_{30}H_{46}O_8$. Here again the substance is a glucoside of an α-desoxysugar and is very easily hydrolyzed by acids. The aglucone which results is sarmentogenin of the formula $C_{23}H_{34}O_5$. The sugar is sarmentose, $C_7H_{14}O_4$, which is isomeric with cymarose and like this is the monomethyl ether of digitoxose or an isomeric α-desoxysugar, $C_6H_{12}O_4$.

The more complex water-soluble but chloroform-insoluble glucosides of *S. sarmentosus* have not been separated as individual substances. Their nature, however, has been explained by the enzyme method. An enzyme prepared from *S. sarmentosus* seeds has been found to hydrolyze the amorphous mixture to glucose and sarmentocymarin. These

glucosides are, therefore, combinations of sarmentogenin with polyoses which in turn consist of sarmentose conjugated with one or more glucose molecules.

Finally, another species of Strophanthus is *S. eminii,* which has been very recently studied by Jacobs and Bigelow. In still unpublished work, the seeds of this species have been found to contain a mixture of easily hydrolyzable and of more stable glucosides. The former, which were not isolated in crystalline form, yield besides strophanthidin, periplogenin, the aglucone of periplocymarin to be discussed below. From the more stable glucoside fraction were isolated a monoside $C_{30}H_{46}O_9$ and a bioside $C_{36}H_{56}O_{14}$. Both of these are glucosides of the same aglucone $C_{23}H_{34}O_5$. The latter could not be isolated as such because of the severe hydrolytic conditions needed for cleavage of the glucosidic linkage. By a special procedure, a trianhydroderivative $C_{23}H_{28}O_2$ was obtained, which proved to be isomeric with a trianhydroperiplogenin produced by similar treatment of periplogenin itself. Although this would indicate that the previous aglucone is not periplogenin but a closely related isomer, it is still possible that periplogenin in stable glucosidic union could give such an isomeric trianhydroderivative, due to an altered course of dehydration. In the above monoside, the aglucone is conjugated with a methyl ether sugar $C_7H_{14}O_5$, either identical or isomeric with digitalose, the sugar of *digitalinum vernum.* In the bioside there is an additional hexose, possibly glucose, attached to this sugar, analogous to the conditions found in the case of K-strophanthin-β already discussed.

The family of *Asclepiadaceae* contains a species *Periploca graeca* from which a crystalline glucoside, periplocin, was isolated by Lehmann (39). The latter showed its glucosidic nature by hydrolysis to an aglucone, periplogenin, and reducing sugar. However, Lehmann's glucoside is only one of a mixture which occurs in the plant. This mixture was shown by Jacobs and Hoffmann (40) to consist of a monoside, periplocymarin, which is present in relatively very small quantities as such, and mostly in combination with one or more molecules of glucose. This mixture was found to yield to the method used for the investigation of kombé strophanthidin. By digestion of the purified extract of the plant with strophanthobiase the crude mixture of glucosides can be hydrolyzed into glucose and periplocymarin. The latter can then be extracted from the complex mixture with chloroform or toluene and crystallizes readily from methyl alcohol with solvent of crystallization. Periplocymarin possesses the formula $C_{30}H_{46}O_8$ and

on hydrolysis gives periplogenin, $C_{23}H_{34}O_5$, and cymarose, the sugar first discovered in cymarin.

The water-soluble periplocin of Lehmann is undoubtedly a bioside consisting of periplogenin in combination with cymarose, which is in turn combined with glucose. Its formula, therefore, should be $C_{36}H_{56}O_{13}$ and not $C_{30}H_{48}O_{12}$ as provisionally adopted by Lehmann at a time when the nature of its sugar component had not been determined.

The *Asclepiadaceae* contain another plant, a species of *Gomphocarpus*, the uzara of certain African tribes, the root of which has furnished a drug "uzaron." According to Henning (41) and Wolff (42) this material contains a glucoside, uzarin, which on hydrolysis gives an aglucone, uzarigenin. Their conclusions as to the formula and properties of these substances have been questioned by Windaus and Haack (43). The latter give the chemistry of this material a different although still provisional interpretation. According to this, uzarin has the probable formula $C_{35}H_{56}O_{16}$ and is a glucoside presumably of an aglucone $C_{23}H_{34}O_5$ combined with 2 mols of glucose. The conditions required for the hydrolysis of uzarin cause decomposition of the genin. The aglucone itself has, therefore, not been described. Windaus and Haack have given an account of a derivative, anhydro uzarigenin, $C_{23}H_{30}O_3$. The reactions of uzarin have definitely shown that its genin is an unsaturated lactone of the strophanthidin group.

Antiaris toxicaria, or the stately upas tree of the Malayan archipelago yields a sap from which two cardiac glucosides have been obtained by Kiliani (44), α- and β-antiarin, to which have been assigned the questionable formula $C_{27}H_{40}O_{10}$ on the anhydrous basis. Both of these substances are glucosides of the same aglucone, antiarigenin, but of different sugars. β-antiarin yields rhamnose on hydrolysis, while the sugar of α-antiarin is antiarose, an isomer of the former. For hydrolytic cleavage such vigorous conditions are required that resinification accompanies the hydrolysis. The yield of antiarigenin is, therefore, small. The formula for this preferred by Kiliani is $C_{21}H_{28}O_5$, but in all likelihood will require revision and probably to a C_{23} basis.

An extremely active crystalline glucoside, convallatoxin, has been isolated by W. Karrer (45) from *Convallaria majalis*, a *Liliaceae*, which possibly belongs to the strophanthus and digitalis group of unsaturated lactones, since it gives the nitroprusside reaction. Nothing further of its chemistry has been reported. There are other active substances in this plant. The so-called convallaramin of Walz (46) is quite certainly not a homogeneous substance. A crude, amorphous, but highly active

substance prepared by Jacobs and Hoffmann (47) and also unquestionably a mixture differs from convallatoxin in that it did not give the nitroprusside reaction.

Squill, or *Scilla maritima* (*Urginea scilla*), also of the *Liliaceae*, is a plant which was used in antiquity as a medicine. Recently a beginning has been made in the study of its chemistry. Stoll (48) has isolated a highly active crystalline glucoside, $C_{36}H_{52}O_{13}$, Scillaren A. A more active and more stable but amorphous glucoside, Scillaren B, which is possibly still a mixture has been but vaguely described. Scillaren A is very labile and is readily hydrolyzed to an aglucone, Scillaridine A, $C_{24}H_{30}O_3$. The latter is really a dehydration product of an aglucone, $C_{24}H_{32}O_4$, which occurs in the glucoside. The sugar is a disaccharide of glucose and rhamnose. The great lability, especially of the crystalline glucoside, has been stated to account for the rapid deterioration of extracts of squills on standing or during previous attempts to isolate the pure principles from it.

From what is known, the aglucones of these glucosides do not belong to the digitalis-strophanthus group. The writer has found that scillaren A and its aglucone do not give the nitroprusside reaction which is characteristic of the latter.

In addition to the plants which have been discussed, there are others which have been reported on one or more occasions to yield digitaloid drugs. Some of these have been listed in the classical paper of Schmiedeberg (49), such as the helleborein of *Helleborus niger*, euonymin, a crystalline glucoside from *Euonymus atropurpurea*, and thevetin of *Thevetia neriifolia*. However, the chemical investigation of these substances has not reached a stage for intelligent discussion. A crystalline glucoside from *Thevetia neriifolia* has been recently but briefly described (50). A so-called alkaloid erythrophleine, a nitrogenous constituent of *Erythrophloeum guineense* or sassy-bark, has been reported to possess a digitaloid action. This material also merits further investigation.

Any account of the digitaloid substances would be incomplete without mention of another group of very interesting substances which are, however, of animal origin—the so-called toad venoms. These substances, which exhibit a potent digitalis action, are secreted by certain (parotid) glands on the surface of these animals. Abel and Macht (51) were the first to report the isolation of a non-nitrogenous, crystalline principle, bufagin, from such a secretion of the tropical toad, *Bufo agua* (*marinus*). In addition a relatively large amount of adrenalin was isolated.

An insight into the chemistry of these substances has been furnished principally by the work of Wieland and co-workers. From *Bufo vulgaris* a substance, bufotalin (52), $C_{26}H_{36}O_6$, was first obtained, which corresponded with but is not identical with bufagin. In later work Wieland and Alles (53) obtained a non-glucosidal, neutral, nitrogenus substance, bufotoxin, $C_{40}H_{62}O_{11}N_4$, which was shown to be a conjugation of bufotalin with argenine and suberic acid. He concluded that bufotalin is a sort of "genin" of the proper toad poison, bufotoxin, in which suberyl arginine parallels somewhat the position of the sugar in the digitalis glucosides. On hydrolysis Wieland showed that bufotoxin, which contains 1 double bond, does not yield bufotalin but a still more unsaturated substance, bufotalein, $C_{24}H_{30}O_3$, along with suberyl arginine and acetic acid. Bufotalin is the acetate of a substance, $C_{24}H_{34}O_5$, which is a lactone and possesses two double bonds and a secondary and a tertiary hydroxyl group. It is, therefore, a derivative of a saturated tetracyclic lactone. Wieland is of the opinion that it will be found to be chemically allied to the bile acids and cholesterol.

In more recent work with the venom of the Japanese toad, *Bufo japonicus*, Wieland and Vocke (54) have obtained gamabufotoxin, $C_{38}H_{60}O_{10}N_4$, which is a lactone and contains 1 double bond. It is also a suberyl arginine derivative, but in this case of a different "genin" than the above bufotalin. Gamabufogenin was isolated in small amount as such from the secretion and has the formula $C_{24}H_{34}O_5$. Direct hydrolysis of gamabufotoxin gives rise to an anhydrogamabufagenin, $C_{24}H_{32}O_4$, which can be isomerized to the same anhydrogamabufogenin which is obtained by the action of strong acid on gamabufogenin. Gamabufogenin is closely related to bufotalin and is also a doubly unsaturated trioxylactone of a tetracyclic system of 24 carbon atoms. But in bufotalin one of the OH groups is acetylated.

Jensen and Chen (55) in a series of studies on Ch'an Su, the dried venom of the Chinese toad, have isolated analogous substances, cinobufotoxin, for which a fomula $C_{43}H_{64}O_{12}N_4$ has been assumed, and a "genin," cinobufagin, $C_{29}H_{38}O_7$. They describe also a *marinobufotoxin*, $C_{42}H_{62}O_{11}N_4$, as the companion of Abel and Macht's bufagin in the venom of *Bufo marinus*. However, very recently (56) Jensen has withdrawn these formulae in favor of $C_{25}H_{32}O_6$ for cinobufagin and $C_{24}H_{32}O_5$ for bufagin. The former should be an acetyl derivative and the latter a formyl derivative of C_{23} genins, which would then place them in relationship to the plant aglucones. These suggestions, however, require careful confirmation.

Further work will undoubtedly prove the well indicated close interrelationship of all of these substances, as well as confirm the belief in a chemical connection with cholesterol and the bile acids. It is not excluded that they may be more widespread in the animal kingdom. Just as adrenalin has been found in a number of these secretions, it is not improbable that these cardiotonic substances occurring here, perhaps also in a perverted rôle, may be found in other animals possessing, as hormones like adrenalin, a normal regulatory function.

II. There is no question that the characteristic pharmacodynamic action of the digitaloid glucosides is directly attributable to a property inherent in the aglucone. This common property, together with certain superficial resemblances noted in the preliminary studies of their chemistry, gave rise to an early suspicion that these aglucones are structurally related.

The following is a list of such aglucones, the formulations of which have been definitely established.

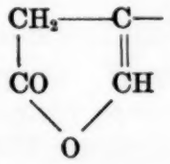
Strophanthidin,	$C_{23}H_{32}O_6$
Digitoxigenin,	$C_{23}H_{34}O_4$
Gitoxigenin,	$C_{23}H_{34}O_5$
Digoxigenin,	$C_{23}H_{34}O_5$
Periplogenin,	$C_{23}H_{34}O_5$
Sarmentogenin,	$C_{23}H_{34}O_5$
Ouabagenin,	$C_{23}H_{34}O_8$

It is probable that others, such as antiarigenin, will be found to possess a similar formula.

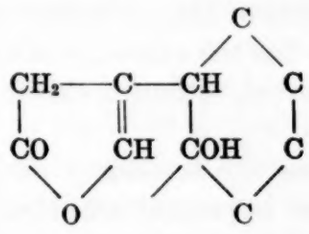
The close resemblance in these formulae is at once apparent. The substances are lactones which consume 1 equivalent of alkali on saponification. In addition each has been shown to possess a double bond which absorbs 1 mol of catalytically activated hydrogen with the formation of a dihydro derivative. Aside from this double bond, they are saturated. They must contain, therefore, 4 rings of carbon atoms. In other words, the aglucones are saturated tetracyclic derivatives. A property possessed in common by the genins and also by the glucosides is the red coloration produced when their solutions are treated with sodium nitroprusside and alkali (57). In addition, they reduce Tollens' solution (57). The source of these reactions has been found to be a common structural feature which involves the association of the double bond with the lactone group.

In a series of studies (58) first on strophanthidin and subsequently on other aglucones, we have shown that each one of these substances is

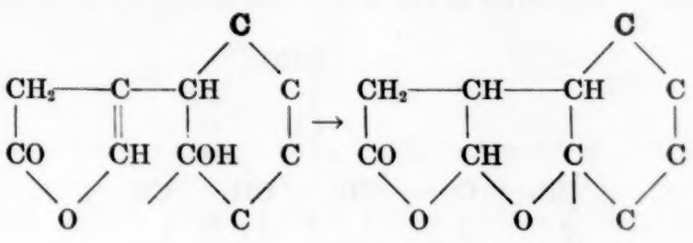
a lactone of a γ-aldehydo acid. They are, therefore, $\Delta^{\beta\gamma}$-γ-lactones, i.e., γ-lactones in which a double bond occurs between the β and γ carbon atoms of the lactone ring, as in the following formula:



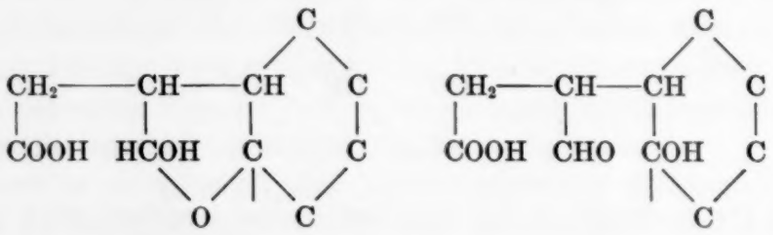
One of the hydrogen atoms attached to the α-carbon atom is active and is responsible for the above reactions. It also reacts with Grignard reagent to give 1 mol of methane. On hydrogenation of the double bond or after saponification this active hydrogen is lost as well as the property to give these reactions. It has also been shown in the case of strophanthidin that the lactone group is a side chain which is attached by its β-carbon atom to a carbon atom of a six-membered ring. In this ring the latter carbon atom is adjacent to another carbon which bears a tertiary hydroxyl group as shown in the following formula:



This configuration of groups has been found responsible for a unique rearrangement which each one of this group of substances undergoes under the influence of alcoholic alkali. An isomerization occurs with the formation of an isoaglucone. Such isoaglucones (isostrophanthidin, isodigitoxigenin, etc.), while still lactones, no longer possess the double bond of the aglucone. Instead a new oxidic ring is formed which involves the tertiary hydroxyl group, a transformation shown in the following formulae:



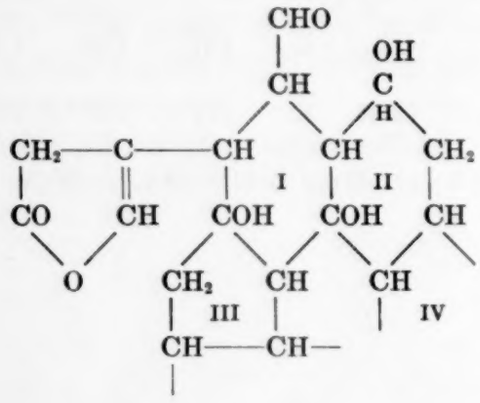
The isoaglucones on saponification yield acids in which the liberated hydroxyl group is the lactol form of a γ-hydroxyaldehyde which can react as such a lactol or in the free hydroxyaldehydic form as follows:



The development of the chemistry of these isoderivatives has been of invaluable service in advancing our knowledge of the structure and interrelationships of the cardiac aglucones.

The aglucones contain other hydroxyl groups. The individual substances differ in regard to the number of and positions occupied by some of these groups. In addition to the above discussed tertiary hydroxyl group which participates in the isomerization to isoaglucones, each has at least one secondary hydroxyl group which is readily acylatable and which is probably the point of glucosidic union with the sugar. Only in the case of gitoxigenin has the existence of more than one secondary hydroxyl group been reported, although this is not excluded in certain other cases.

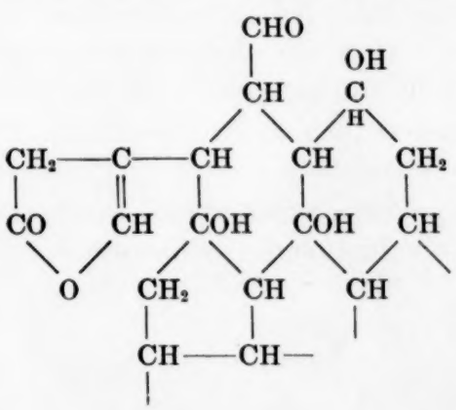
Strophanthidin possesses one secondary and two tertiary hydroxyl groups. In addition there is present an aldehyde group. In a series of studies it has been possible to determine the location of these groups. The two tertiary hydroxyl groups and the aldehyde group are attached to the same six-membered ring which carries the unsaturated lactone group. Adjoining this ring is a second ring which carries the secondary hydroxyl group. This second ring has also been found to be six-membered. Finally, in very recent work Jacobs and Elderfield (59) have made observations which permit the conclusion that a third ring is five-membered. These observations are best presented in the following graphic formula which is not entirely complete and in which the rings are designated in the order of the above discussion by Roman numerals:



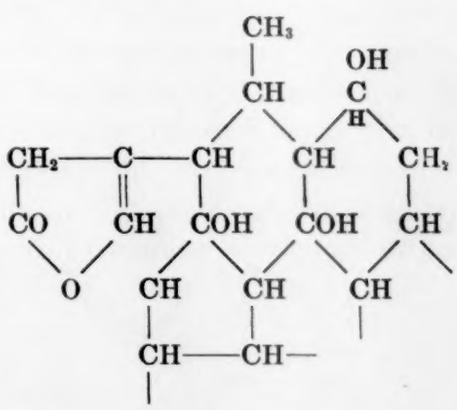
The position and size of the fourth ring have not yet been determined. There is good reason to believe that it is attached to ring II and is five- or six-membered.

As a result of another direction which the investigations have taken, our knowledge of the structure of strophanthidin has become applicable to the structure of other aglucones. There has naturally been the belief that digitoxigenin and gitoxigenin occurring in the same plant must, therefore, be closely related. Attempts, although unsuccessful, have been made by Windaus and co-workers (60) to demonstrate this fact by their transformation into an identical saturated desoxylactone. Although the chemical transformations were made, the substances proved to be not identical but isomeric. This correlation has been accomplished by Jacobs and Gustus (61) by the use of a different procedure in which the isoaglucones were employed. By transformation through individual series of derivatives in each case isodigitoxigenin and isogitoxigenin were converted into an identical substance. This result permitted the conclusion that gitoxigenin differs from digitoxigenin by the possession of one extra secondary hydroxyl group to which a definite position can be assigned. Gitoxigenin is, therefore, hydroxydigitoxigenin. In a separate series of studies Jacobs and Elderfield (62) have shown that a derivative of strophanthidin, isostrophanthidic acid, can be transformed by the Wolff method into the desoxo derivative, i.e., the aldehyde group as it occurs in strophanthidin can be reduced to methyl. This desoxo compound proved to be isoperiplogenic acid, a periplogenin derivative. Periplogenin differs from strophanthidin only by its possession of a methyl group in place of the aldehyde group of the latter. It is, therefore, desoxostrophanthidin.

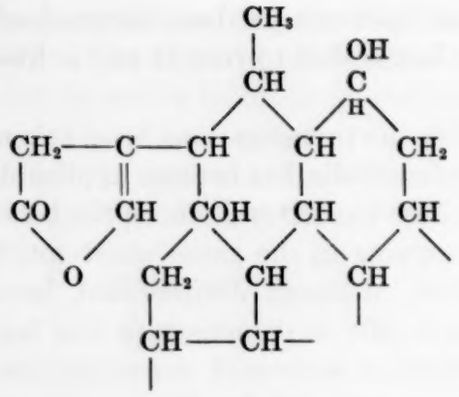
It was then found possible by a further series of reactions starting from the above mentioned isoperiplogenic acid to achieve a substance which could also be prepared from digitoxigenin (63). This permitted the structural correlation of digitoxigenin, and therefore of gitoxigenin, with both periplogenin and strophanthidin. These relationships are presented in the following partial formulae:



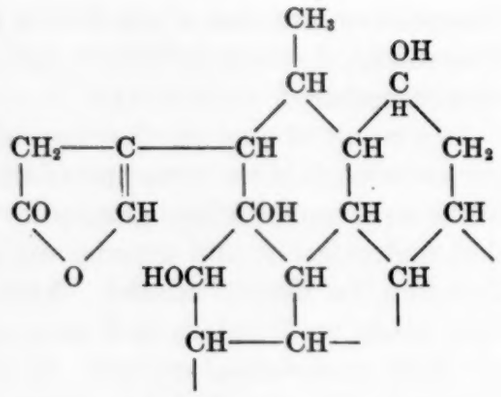
Strophanthidin



Periplogenin



Digitoxigenin



Gitoxigenin*

* More recent work of JACOBS AND ELDERFIELD (Journ. Biol. Chem., 1933, xcix, 693) has shown that another position will have to be assigned to the extra hydroxyl group of gitoxigenin than that given in this formula.

There is now very good reason to believe that the third aglucone, digoxigenin, will have the same general structure and that it will be a hydroxydigitoxigenin. Its isomerism with gitoxigenin is probably due to the position occupied by the extra hydroxyl group. The same quite possibly obtains in the case of sarmentogenin. Similarly, the aglucone of ouabain is probably a polyhydroxydigitoxigenin. In antiarigenin a carbonyl group is also present. From the preliminary observations of Kiliani, the latter appears to be ketonic in character and not aldehydic as we have found to be the case in strophanthidin.

Jacobs and Hoffmann (30) have shown that in cymarin the sugar, cymarose, is attached to the secondary acylatable hydroxyl group of strophanthidin. It is most probable that this is the point of conjugation in the case of the other aglucones, although in some cases other hydroxyl groups may be considered as auxiliary points of conjugation. This is a question which requires further investigation.

Since the pharmacology of the digitaloid glucosides as a group was developed before any real progress in its chemistry was made, biological procedures have naturally been the main reliance in the assay of the various substances, tinctures, infusions, etc. No attempt will be made to discuss such methods in this review. However, an interesting chemical method has been developed by Knudson and Dresbach (64) which was first applied to digitalis extracts and then to strophanthus. This method was based on the original empirical observation of Baljet (65) that cardiac glucosides of digitalis and strophanthus and their genins give an orange-red color with dilute alkaline picric acid solution. The

method of assay consists briefly in decolorizing the solutions with lead acetate and after removal of excess lead in treating the solution further with an alkaline picric acid solution. The amount of digitalis or strophanthus glucosides can be calculated after comparison of the color intensity with that given by a standard ouabain solution or with a permanent standard consisting of a solution of potassium bichromate.

The present writer has found in unpublished work that this reaction is due to the same cause which gives rise to the nitroprusside reaction, i.e., the active hydrogen of the unsaturated lactone group characteristic of the aglucones of these glucosides. The dihydro glucosides and the dihydro genins as well as the isogenins no longer give the Baljet reaction. The reaction is also given by the simpler $\Delta^{\beta\gamma}$-unsaturated lactones such as the angelicalactones which still have a hydrogen atom attached to the α-carbon atom. This method, therefore, is a rational chemical method of assay and some of the experience has shown it to give results comparable with those of the biological procedures.

However, caution must be used in the case of the assay of aqueous infusions of seeds and possibly of leaves. As already discussed, the writer has found that if a mash of defatted *S. kombé* seeds is allowed to stand, the glucosides are transformed into allocymarin which is practically devoid of the characteristic cardiotonic activity. This glucoside as well as its aglucone still give the characteristic Legal and Baljet reactions.

It is interesting to speculate on the relationship between molecular structure and the cardiotonic action of this group of substances. There is no question that the latter is a quality inherent in the aglucone molecule itself. Our present knowledge does not point to any particular group in the aglucone which is directly responsible but to the molecule as a whole. However, a few observations have been recorded which show the influence of certain chemical changes on the action. For example, after hydrogenation of the unsaturated lactone group, the action is greatly reduced or perhaps eliminated. Dihydroouabain is roughly $\frac{1}{16}$ as active as ouabain. Dihydrocymarin is only $\frac{1}{23}$ as active as cymarin (66). Dihydrodigitalin proved to be non-toxic (67) in amounts which were 12 times the toxic dose of digitalin. A similar conclusion was reached by Cloetta (68) with the digitalis genins. However, in allocymarin which still possesses this group the toxicity has been perhaps entirely removed. On isomerization to isostrophanthidin, the cardiac action of strophanthidin has been found by Straub (69) to be practically lost. Similarly, saponification of the lactone group of cy-

marin to a cymarinic acid caused a reduction in the activity to 1/500th. In view of later chemical developments, the identity of this so-called cymarinic acid is in question. An older observation of Kiliani and Windaus (70) may be cited, on so-called digitalein. Solutions of this material became acid on standing, due to the opening up of the lactone group with the formation of a biologically inactive acid. Similar observations have been reported by Brauns and Closson in the case of so-called crystalline K-strophanthin (71).

In this connection an important point is raised as to the reason for the frequently observed deterioration in strength of solutions of the various digitalis, strophanthus and other preparations. Such deterioration when not due to the obscure effects of sunlight, molds, bacteria, etc., can fairly readily arise from chemical changes which involve the lactone groups of the component genins. These groups may gradually open with the formation of practically inactive acids or perhaps isomerization may occur. Factors which favor such changes are a change in reaction of the solution towards the alkaline side, perhaps caused by the alkali of the glass container and increased temperatures. To counteract this effect Levy and Cullen (72) have made use of a phosphate solution in the case of ouabain.

Finally, although from a qualitative standpoint the cardiotonic actions of the natural glucosides are roughly a common one, certain differences have appeared in the measurements of their effects. In general, whereas cymarin and ouabain are of a similar general order of toxicity for frogs, that of digitalin and digitoxin has been reported to be about $\frac{1}{4}$ to $\frac{1}{8}$ of their toxicity. A recent statement (73) gives to lanadigin about $\frac{1}{10}$ the toxicity of ouabain. On the other hand, digoxin, which possesses the same aglucone but a different sugar moiety, is apparently $2\frac{1}{2}$ times the toxicity of lanadigin (74). It appears, therefore, that the sugar portion of the molecule, while itself toxically inert, plays some rôle in influencing the character and relative intensities of the cardiac effects, perhaps by virtue of its effect on the solubilities, diffusibilities, and therefore on the absorption and transport of the effective agent. Since the aglucones themselves are closely allied and differ apparently almost entirely by the number and positions of the substituting hydroxyl groups in their molecules, it is obvious that this difference in structure also contributes to the differences in toxicity noted.

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TRANSMISSIBLE TUMORS OF THE FOWL

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It has long been known that neoplasms occur in fowls, and a variety of types was described in the early literature on animal tumors. Transplantation of the avian growths was not accomplished until 1910, when Fujinami and Inamoto succeeded in inoculating a myxosarcoma, and later in the same year Rous reported the transmission of a spindle-cell sarcoma. In the following years more than forty spontaneous tumors of the fowl were investigated in the laboratories of the Rockefeller Institute, and four of them proved transplantable. Other laboratories in turn reported additional examples of the transmissibility of fowl tumors. Up to the present time something over twenty-seven tumors representing a variety of types have been transplanted and made the subject of investigation.

In 1911 Rous demonstrated that Chicken Tumor I could be transmitted by a cell-free filtrate and Murphy succeeded in transferring the tumor with desiccates. Since then this property of transmission by agents other than living tumor cells has been demonstrated for practically all of the other transplantable chicken tumors which have been carefully investigated, and is accepted as characteristic of the group. This discovery incited considerable controversy regarding the significance of this pathological group. The first and perhaps the logical deduction was that the filterable agents were filter-passing parasites—a conclusion which led many to consider the chicken tumors as granulomas unrelated to true neoplasms of mammals. Supporting this attitude was the fact that years of intensive study of the mammalian tumors had failed to furnish any evidence of a parasitic cause. However, more complete investigation of the chicken tumor group showed that histologically and in their general biology there was no essential property except transmission by agents other than tumor cells which differentiated the avian from the mammalian tumor. At present there is a fairly universal acceptance of the chicken tumors as true neoplasms.

The transmission of fowl leucemia by means of cells and by cell-free filtrates was demonstrated somewhat earlier (1908) than the transmis-

sion of chicken sarcoma. While leucemias have been referred to frequently as neoplasms of the hematopoietic system the full significance of the transmission of fowl leucemia from the point of view of malignant disease in general was not appreciated until recently. The close relationship between leucemia and tumor in mice is indicated by the work of MacDowell and Richter, who have shown that the occurrence of the spontaneous diseases are equally dependent on certain inherited tendencies. Transmission in both instances is accomplished only by living cells and is definitely limited by genetic factors. Leucemia and tumors of fowls have the property in common of being transmissible by agents separable from the cells and recent work by Furth has shown a close similarity in the properties of the transmitting agents of the two disease groups. It seems therefore now that transmissible leucosis of fowls should be definitely included in a discussion of transmissible avian tumors.

Tentatively accepting the conclusion that fowl tumors are true neoplasms, as most investigators have, the main discussion centers around the nature of the transmitting agents. The popular interpretation is that these agents belong to the filterable virus group. On the other hand a few investigators have felt that the properties of the agents indicate that they belong to a different order, representing perhaps a previously unrecognized type of mechanism. Considerable investigation has been carried out to test this latter point of view.

We shall not include in the present paper any detailed account of the individual tumors, but shall attempt to present principally the properties of the tumor agents, which may throw some light on their nature.

GENERAL CHARACTERISTICS OF THE TRANSPLANTABLE CHICKEN TUMORS. With the exception of two, the chicken tumors which have been transmitted belong to the sarcoma group. A list of these is given in the table on page 248. The more common type is histologically a spindle-cell sarcoma with more or less of a tendency towards a myxomatous transformation with the production of a mucoid exudate.

Tumors of this character, successfully transplanted, metastasize freely to organs and tissues, and may cause the death of the fowl in two to five weeks. With desiccate or filtrate there is a latent period, but after the new growth appears it develops with a vigor quite equal to that of tumors resulting from inoculation of cells. The other extreme from this highly malignant type is found among the fibromas or fibro-sarcomas where the growth rate is very slow and metastases are rare. Among the group are an osteochondrosarcoma, a teratoma and an endothelioma. Besides

Transplantable tumors of the fowl

DENOMINATION	DESIGNATION	DATE OF DESCRIPTION	TRANSMISSIBLE BY FILTRATE	TRANSMISSIBLE BY DESICCATE	NATURE
Ellermann and Bang		1908	+	+	Transmissible leucemia of fowls (leucosis)
Fujinami and Inamoto	I	1910	+	+	Myxosarcoma
Rous	I	1910	+	+	Spindle-celled sarcoma
Rous, Murphy and Tytler	VII	1912	+	+	Osteochondrosarcoma
Rous and Lange	18	1913	+	−	Sarcoma of intracanalicular pattern
Inamoto		1913	?*	?	Myxosarcoma
Rous	38	1914	+	+	Spindle-celled sarcoma with blood sinuses
Lange	43	1914	+	+	Spindle-celled sarcoma
Muto		1915	+	+	Osteochondrosarcoma
Hayashi		1915	+	+	Fibromyxosarcoma
Fujinami and Kato	II	1915	−	−	Fibroma
Pentimalli		1916	+	+	Sarcoma
Fujinami and Kato	III	1916	?	?	Chondroma
Yamamoto		1918	?	?	Spindle-celled sarcoma
Oshima		1919	?	+	Sarcoma
Teutschlaender	I	1921	+	+	Sarcoma
Kubo		1923	?	?	Fibroma
Roffo		1925	−	?	Round-celled sarcoma
Peyron, Thomas and Surmont		1926	?	?	Sarcoma
Begg	I	1927	+	+	Fibrosarcoma
Begg	II	1927	+	+	Endothelioma
Teutschlaender	II	1929	+	?	Sarcomas
Teutschlaender	III		+	?	
Teutschlaender	IV		+	?	
Baker		1931	?	?	Teratomatous ovarian tumor
Andrewes	RF 4	1932	+	?	Spindle-celled sarcoma
Andrewes	BS 1	1932	+	?	Spindle-celled sarcoma
Andrewes	RF 11	1932	+	?	Fibrosarcoma

* ? = No record.

the variety of histological types, which are just as faithfully transmitted by the agents as by the cells, the tumors also show distinct variations from each other in their general biological behavior. Two striking examples of this from the Rockefeller Institute series are tumors nos. 18 and 38, which showed a distinct tendency to metastasize to the

skeletal muscle without involvement of the lungs—the favored location for metastases of the majority of other tumors.

The early study of these growths not only showed histological structures similar to mammalian tumors but the general behavior of the inoculated tumors was similar. The spontaneous tumors appear to develop in a single focus, and from this point invade the surrounding tissue. No contagion has been noted even when normal susceptible fowls have been kept in close contact for long periods with tumor-bearing chickens. There is evidence that the tumors resulting from inoculation of tumor cells are formed by the multiplication of the individual cells which actively invade and replace the normal tissue. Metastasis generally takes place through the blood stream, more rarely by the lymphatics, and there is support for the belief that the secondary growths are the result of dislodged cells from the primary tumor. In susceptible fowls there is an absence of any significant reaction about the tumor, but a graft introduced into a resistant chicken calls out a type of reaction characteristic for mammals under similar conditions. Both natural and acquired resistance to the tumor are observed and in the latter type the retrogressive tumor shows a general histological picture similar to that seen in retrogressing tumors in mammals. The individual tumors represent fixed types and little variation, except for changes in rate of growth, is noted after many generations of transplantation. There is a greater susceptibility of young birds, and those in poor physical condition are less receptive than healthy fowls. In all these respects the avian tumors resemble closely the mammalian type. For a more detailed description of the histology and general characteristics of these tumors the reader is referred to the original articles, many of which are illustrated.

Species specificity. Until recently it was thought that chicken tumors could not be transferred beyond the species. In fact, Chicken Tumor I represented in its early history a striking example of species limitation in that at first it was transplantable only to blood relations, later only to a special breed of fowl, and even after some years of propagation the inoculation into foreign species yielded no result. While the other tumors did not show the same limitations as far as breed of chickens was concerned, the attempts to transfer them to other birds or mammals failed. Within the last few years Fujinami and Suzue (1928), working with a highly malignant strain of chicken myxoma, succeeded in growing the tumor in ducklings, and later reported that after 40 generations in the duckling the tumor could be readily returned to chickens. Des

Ligneris (1930) succeeded in transplanting Chicken Tumor I in turkeys and guinea fowls, but he was not able to induce tumors with filtrates nor did he carry the tumor beyond the third generation in the foreign species. More recently, Gye has confirmed the observations of Fujinami and Suzue with the myxoma and has further claimed to have induced tumors in ducklings with filtrates. Andrewes, working with Chicken Tumor I (1932) has described positive inoculations in young pheasants with both tumor cells and filtrates.

The significance of these observations has not yet been fully taken into consideration. Formerly, with mammalian tumors the idea of species limitation of transplantation was so firmly fixed that in two instances where the limitation did not hold the growths were automatically classed as granulomas. Recently, however, Cloudman (1932) has obtained progressive growth of a mouse tumor transplanted into mice of different species but belonging to the mouse genus. According to these results, the interspecies transplantation of malignant tumors does not seem to be confined to birds, and in this respect, the differences between birds and mammals exist only in the narrower range of the species limitation for mammals.

GENERAL PROPERTIES OF THE TRANSMITTING AGENTS. As by far the major part of the investigation on the nature of the tumor agents has been carried out on that of Chicken Tumor I, and as the others have shown no essential variation, the experimental data derived from its study will be chiefly considered in the following discussion.

Resistance to various physico-chemical agents. The agent kept in solution gradually loses its tumor-producing activity. At room or incubator temperature it generally becomes inactive in 24 or 48 hours, but if kept at the freezing point it may retain its activity for a considerably longer period. This spontaneous degradation has been ascribed to oxidation on the basis of the experiments of Mueller, who found that the addition of cystein hydrochloride to a filtrate preserved it active over a significantly longer period. Gye and Purdy accepted a similar interpretation to explain the protective action of hydrocyanic acid.

The agent is rapidly destroyed in autolyzing tissue, but if the tumor is desiccated *in vacuo* at low temperatures the activity will be retained indefinitely. In the dry state the agent will withstand absolute alcohol, toluol or acetone over long periods, but is destroyed by dilute alcohol, 0.5 per cent phenol, bile, saponin, chloroform and a variety of other substances. Repeated freezing and thawing of the tumor tissue has little or no deleterious effect on the agent.

While the chicken tumor cells are killed by heat and ultra-violet light quite as readily as normal cells, the tumor agent is more resistant. It will withstand a temperature of 50°C. for 15 minutes, but at 55°C. is inactivated. A large number of bacteria and viruses tested show about the same degree of susceptibility to various wave lengths of ultra-violet light, while the tumor agents are not only far more resistant but show a different relative susceptibility to selected wave lengths. The variation in hydrogen ion concentration tolerated by the tumor agent ranges from pH 4 to pH 12. The acid reactions seem more harmful.

Size of the transmitting agent. While methods of estimating the size of filterable particles are not accepted as entirely satisfactory, there have been attempts to arrive at some figure for the tumor agent. The first rough study indicated that the agent would pass Berkefield filters V and N but rarely W and not at all Chamberland F. By use of ultra-filtrate methods the size has been variously estimated by different observers as 10 to 100$\mu\mu$. Most of these studies have been made with crude tumor extracts and filtrates, and as yet there have been no tests with the purified agent free from contaminating protein.

Tissue affinities. It was evident early in the study of this group of tumors that the agents were confined in their action to specific cells. Filtrate injected into developing chick embryos or their membranes failed to induce tumors except where the agent came in contact with mesodermal tissue. Injections of cell-free tumor extracts into the breast muscle of adult fowls induced tumors only along the path of injury inflicted by the injecting needle, regardless of the amount of filtrate injected. Intravenous injection of the agent induced tumors only at sites of some tissue derangement, e.g., actively functioning ovary. This suggested at first that injury to the cell rendered it more susceptible, but later work, together with the observations on the embryo, indicated that young or dividing cells are more easily affected. As the agents from sarcomas have a selective action on connective tissue cells, so the filtrate of an endothelioma acts on endothelial cells. Besides this *in vivo* affinity, there is evidence that certain tissues adsorb and fix the agent *in vitro*. Pentimalli observed that chick embryo pulp or young tissue from a healing wound definitely adsorbed the agent from a tumor filtrate. Duran-Reynals and Murphy have demonstrated that muscle from a susceptible fowl adsorbs and fixes the agent of Chicken Tumor I *in vitro*, while similar tissues from non-susceptible species are without effect. Furthermore, they showed that non-mesenchymatous tissue from susceptible fowls had no binding property for the agent. As a

contrast to these results, viruses having definite selective action on certain tissues or cell types are not bound or inactivated by *in vitro* contact with these tissues or cells.

ANTIGENIC PROPERTIES OF THE AGENT. The early attempts to produce protective antibodies in chickens by injection of attenuated agent yielded little of significance, nor did the transfusion of large amounts of blood or serum from resistant fowls influence the growth of established tumors in susceptible ones. Some results indicate that two types of immunity exist, one directed against the etiologic agent as distinct from that directed against the malignant cell. Mottram found evidence of antibodies in a few fowls in which the tumor had retrogressed. Later Andrewes demonstrated that chickens bearing slow-growing fibrosarcomas after some months developed in their blood neutralizing bodies effective against more rapidly growing tumors. According to him similar neutralizing factors are occasionally found in the sera of normal fowls. It has been suggested that these factors may be "inhibitor" of the type described from this laboratory, rather than antibodies. The first attempts to study antibody formation in foreign species was complicated by the fact that the tumor agent was not freed from the incidental tissue anb blood components. Rous, Robertson and Oliver, while they failed to get clear evidence of antibody formation in rabbits, did succeed, as the result of injection of tumor tissue and blood of fowls in the last stages of the disease, in developing neutralizing bodies in the serum of geese. Jashikawa obtained neutralizing serum from rabbits which had been injected repeatedly with chicken tumor extracts. Mueller, using a suspension of tumor cells, induced antibodies to the agent in rabbits and ducks, while Gye and Purdy succeeded in accomplishing this with tumor filtrate in both ducks and goats. Recently a report was made from this laboratory in which an account was given of the antigenic properties of the tumor agent tested in various stages of purification. We confirmed the observation that effective precipitating and neutralizing antibodies may be developed in the rabbit with concentrated filtrate of Chicken Tumor I. Similar results were obtained also with an active protein fraction of the filtrate. After the tumor agent is separated from the major part of the incidental proteins, a procedure which can be accomplished without detectable loss in activity, it will still call out neutralizing antibodies in rabbits, but the serum shows no evidence of precipitins. After further removal of impurities (mucoid material) there seems to be a complete loss in antigenic properties. Rabbits injected repeatedly with this material not only failed to show precipitating and neutralizing

antibodies in their serum but complement fixation tests were also negative. Furthermore, injection of larger amounts of this material failed to induce any sensitization in guinea pigs to a second injection.

It is suggested that the precipitins are developed against the incidental tumor and tissue proteins and are not involved with the immune bodies capable of neutralizing the tumor agent. There seems little doubt that a sufficient amount of agent is present in the purified product to induce antibody formation if the agent in this condition possesses antigenic properties. No satisfactory explanation of this result is evident at the moment.

INHIBITORS ASSOCIATED WITH THE TUMOR AGENT. On the assumption that the tumor agents may be of endogenous origin representing abnormal activities of the forces which normally cause growth and differentiation of tissues, it was suggested some time ago that if such be the case there should exist factors which tend to balance and control these forces. The first evidence that such an inhibitor exists in the tumor was found in the course of an investigation of the fact that occasionally an extract of a tumor desiccate failed to induce tumors, while injection of the residue showed that it contained a considerable amount of the agent. It was noted that if such a residue was extracted a second time this extract was capable of inducing tumors. The residue left from this, extracted a third time, yielded a still more active product. Inoculation of samples from the residue after each extraction showed their tumor-producing properties progressively increased. This result was interpreted as indicating that an inhibiting substance somewhat more soluble that the agent was present in some tumors, and on account of its solubility comes out in the first extract, thus accounting for the absence of activity, or low grade activity of the first extracts.

Substantiation of the above conclusion was shown by two other experiments. Tumor filtrates or extracts of desiccate of low grade activity, treated with an amount of type C aluminum hydroxide and then separated from the aluminum, has a tumor-producing activity far greater than the untreated material. This indicates that the low order of activity of these extracts is not due to the absence of the agent but rather to the presence of something that interferes with the activity of the agent. More direct evidence of this is found in the fact that these extracts of slow-growing tumors, heated to a point where the tumor agent is inactivated, are capable of neutralizing the tumor-producing property of highly active tumor filtrates. Furthermore, the aluminum hydroxide used to adsorb out these low grade extracts will yield under

treatment with alkaline solutions the inhibiting factor in considerable concentration, capable of neutralizing the tumor-producing agent in its most active form.[1]

In addition to the experiments quoted above indicating the presence of an inhibitor, Gye and Purdy have drawn a similar conclusion from some of their experiments, and Sittenfield, Johnson and Jobling, besides demonstrating an inhibitor in the tumor, report its presence in the blood of both tumor-bearing and normal chickens.

PROGRESS IN THE ISOLATION OF THE ACTIVE PRINCIPLE FROM CHICKEN TUMOR EXTRACT. The process of filtration through Berkefeld candles, which has been successfully applied to the separation of an independent causative agent from the tumor cells, constitutes also an important means of purification, in the fact that it yields a fluid free from tissue debris and the larger insoluble particles.

The filtrate of Chicken Tumor I is a clear fluid, more or less colored by blood pigments, and usually presents a marked viscosity. When the extraction has been performed with distilled water the reaction of the extract is around pH 6.6. Addition of salts or of alkali increases the solubility of the tumor material. Tumor filtrates subjected to increasing acidity show gradual development of opalescence and finally a precipitate separates when the reaction of the fluid attains pH 4.8 or when the corresponding value of the hydrogen ion concentration lies below this point. Such a precipitation can be obtained by any means which provides the adequate acidity. Mineral or organic acids, buffered solutions, removal of cations by means of electrodialysis, bubbling through of CO_2 have been used with similar results. The collected precipitate usually carries down the activity, provided the reaction has not dropped below pH 4.0, at which point the tumor agent is inactivated. The presence of the tumor agent with the precipitate must be ascribed to an adsorption phenomenon, for when a precipitate occurs in an extract the active principle has a tendency to follow and the adsorption is apparently not specific. Protein fractions active in producing tumors have also been isolated by other means such as precipitation by ammonium sulfate at half saturation. However, other methods of purification have shown that these fractions were mainly unrelated contaminating material.

[1] The absence of species limitation in the action of the inhibitor from the chicken tumor and the suggestion that there is a tissue specificity is indicated by the fact that the inhibitor has a definite action on a mouse sarcoma and not on a carcinoma. (Murphy and Sturm, 1931, 1932.)

Purification by adsorption. Leitch was the first to apply the method of adsorption and elution to the study of chicken tumor material. In this respect different applications of the method of adsorption are possible, depending on whether one aims to absorb the principle to be isolated and to release it subsequently, or to adsorb and eliminate contaminating substances from the fluid. According to the sort of adsorbent which is used one or the other end may be obtained but usually there is no sharp difference between the two, the amount of adsorbent used more or less determining the result. Kaolin, alumina gel and charcoal have been studied up to the present time (Fränkel, Lewis), with somewhat similar results. When chicken tumor material is treated with a proper amount of aluminum hydroxide (Willstätter type C), the fluid which is left behind retains the property of inducing tumors at least to the same extent as that exhibited by the original material. By this method the tumor agent is freed of much of the contaminating proteins and incidentally the aluminum also removes the inhibiting factor, thus leaving the tumor agent in a more active form.

The purified fraction is a clear and colorless fluid, which still exhibits the characteristic viscosity of the plain tumor extract. Chemical tests performed on this material reveal that practically all proteins have been removed. However, the ninhydrin test and the diazo reaction of Burian remain positive. Free reducing substances are absent, but the Molisch test and the naphthoresorcinol test for glucuronic acid are positive. Hydrolysis with strong acids yields a considerable amount of reducing material. The nitrogen content has been reduced to a marked degree when the starting material is an extract of dry powder and even much lower if the extract used is prepared from fresh tumor tissue.

This purified fraction submitted to biological tests proved it to be lacking in the ordinary antigenic properties which are possessed by the crude tumor extracts. Repeated injections into rabbits failed to induce the production of precipitins or sufficient antibodies to fix the complement in the Bordet reaction. The material failed to induce anaphylactic symptoms in sensitized guinea pigs. However, the purified fraction retains the property of inducing neutralizing antibodies when injected into rabbits.

Method of elution. The method of adsorption and elution widely applied by Willstätter to the concentration of enzymes has failed with the chicken tumor agent in the sense that no enrichment of the active principle could be obtained. The concentrated eluted fraction is usually more or less colored by released blood pigments and tests for pro-

teins are positive. The nitrogen is comparatively higher and, moreover, the tumor-producing activity of the released fraction is much inferior to and more irregular than that exhibited by the original extract or the so-called purified fraction. On the other hand, it has been shown that an inhibiting substance was adsorbed on alumina gel and when released from its union with the adsorbent, exhibits high neutralizing powers for the chicken tumor principle.

Precipitation with gelatin. On the basis of chemical tests and analyses it was assumed that material found in abundance in the "purified fraction" and yielding large amounts of reducing substances on hydrolysis was chondroitin-sulfuric acid or a substance of a related nature. A special method was adapted for the removal of this material in order to ascertain its hypothetical relationship to the chicken tumor principle. If a certain amount of gelatin be added to the already purified fraction and the reaction of the mixture brought to pH 4.7 by the addition of a buffered solution, a flocculent precipitate forms which can be removed by centrifugation. The supernatant fluid is clear and colorless, is deprived of viscosity and the tests for glucuronic acid and carbohydrates are negative. It no longer precipitates on addition of basic lead acetate. In spite of the successive manipulations to which it has been subjected, the fluid, finally purified by gelatin, retains a major portion of the tumor-producing activity of the starting material. It fails to produce anaphylactic shock in guinea pigs or precipitins in rabbits. There is evidence that practically no neutralizing antibodies develop in animals repeatedly injected with this material.

Purification by dialysis.[2] Additional purification of the tumor extract can be obtained by submitting it to dialysis in collodion bags against running distilled water. By this method, the fluid can be rid easily of the substances which in the first fraction are responsible for the ninhydrin test and the diazo reaction without materially reducing the tumor-producing activity. Compared with the fraction purified by adsorption, 70 per cent of the solids are removed by the process of dialysis. The nitrogen content can be reduced to 29 per cent of its original value. The phosphorus figures, however, remain unchanged. With material prepared from dry tumor, the nitrogen content of the finally purified fraction may be as low as 0.01 mgm. per cubic centimeter, or about 7 per cent of dry weight.

To summarize, it is demonstrated that the causative principle of chicken sarcoma can be obtained in purified solutions potentially as

[2] Unpublished experiments.

active regarding their powers of inducing tumors as the crude original extract.

In contrast with this practically unreduced activity, usual chemical and biological tests for proteins have become negative. An important contaminating substance, a carbohydrate, can be removed without markedly reducing the activity. A certain number of remaining impurities can be eliminated by further manipulations and, according to corresponding observations, the chicken tumor principle does not appear to give positive tests such as the ninhydrin test or the diazo reaction. Moreover, the agent is not affected by basic dyes such as neutral red, by barium chloride, and is not precipitated by basic lead acetate.

Discussion. When evidence was first presented that a series of chicken tumors could be transmitted by agents separable from the cells, pathologists hesitated to accept the new group as true neoplasms. However, closer study of the fowl tumors revealed that the principal characters recognized as typical for the transplantable mammalian neoplasms, especially sarcomas, were reproduced by the fowl disease, with the additional property of transmissibility by filterable agents. The failure so far to demonstrate such an independent etiological agent in mammalian new growths may be due only to inadequate methods of extraction. Certainly such a negative result with the latter tumors is not sufficient ground on which to exclude the fowl disease from the group of malignant diseases. The fact that two of the older chicken tumors have been transmitted recently to other species of fowl may be considered by some as sufficient reason for the segregation of this group, but in our opinion this fact has not been investigated sufficiently to justify an estimate of its importance at the present time.

Although a tacit agreement may have been reached regarding the neoplastic nature of the chicken tumors, the nature of the transmitting agent or agents remains as yet unsettled. The general tendency has been to class them among the filterable viruses. Unfortunately the so-called filterable virus group has been used to a considerable extent for the indiscriminate segregation of disease-producing agents of unknown nature, having in common principally their sub-microscopic size and the fact that they appear to be obligatory parasites of living cells.[3] The group has included at times such unrelated agents as the filterable organism of pleuropneumonia and the principle causing mosaic disease of plants. The former was eliminated from the group because it was

[3] In recent publications by G. H. Eagles and co-workers claims are made that vaccine virus can multiply in cell-free medium.

cultivated on a cell-free medium and could be demonstrated under the microscope, and the latter agent, according to the recent work, is probably an inanimate chemical substance. However, there exists among the so-called viruses a number of infective agents having much in common, which are frequently referred to as typical viruses, and considered by most investigators to be ultra-microscopic formed organisms.

In common with the so-called viruses, the tumor agents are ultramicroscopic, filterable and their proliferation is intimately dependent on the presence of susceptible living cells. On the basis of such very general characters one might be justified in including these agents among the viruses. If Chicken Tumor I were the only type of tumor transmitted by a factor separable from the cell it would not be difficult to accept the idea of a living virus as the causative agent. But it is not easy to expand the theory to explain the variety of types of chicken tumors, each with an agent, not only limited in its action to a definite cell type but after forming its contact with the cell, causing it to grow and differentiate into a specialized type. For the chicken tumor group alone we would have to consider that there are large varieties of viruses, and if the theory be extended to other neoplasms to explain their fixed individual histological and biological characters we would almost have to suppose a separate variety of virus for each tumor. This has been the principal argument against the virus theory for tumors. Gye in his attempt to get around this difficulty devised a two-factor theory, suggesting a single virus type as the principal causative agent and a second factor, derived from the infected cell, combining with the virus and giving it the extraordinary specificity. The experimental evidence brought forward to support this idea has failed to be confirmed by most of the experienced investigators. The basis for this theory is not sufficient at the present to require its detailed consideration. In any discussion of the nature of the tumor agent consideration must be given to such properties as their species and cell specificity, the multiplicity of tumor types produced, each with its individual agent, the fixation of the agents by a substrate (i.e., the protein of susceptible tissues), the characteristic reaction to ultra-violet light, and the fact that it may be dissociated from the tumor proteins and brought to a degree of purification without loss in activity, showing considerable resistance to chemical manipulation, the association with a balancing or inhibiting factor, and the peculiar antigenic properties. While these characteristics are different from those of the typical viruses it is probable that no one of them alone definitely separates the two groups; together, however, they are more than just indications of an essential difference.

We believe that the facts which have been reported are in favor of the hypothesis that the specific principles of chicken tumors are comparatively simple substances which belong to the field of biochemistry rather than to that of bacteriology. Regarding the mechanism of action and the production of the chicken tumor agents in susceptible tissues two main theories have been presented. A certain number of workers consider the active principle to be in the nature of an enzyme, the cause of the specific cellular disturbance which would in turn be produced by the diseased cell. In this case, one must imagine an initial rupture of the cell's stability induced by an unknown factor in favor of a particular endogenous enzyme with the resulting modification of the cellular metabolism: the disease. The excess of enzyme produced could be separated from the originating element and, brought in contact with normal susceptible cells, would reproduce a similar unbalanced metabolism. The term of enzyme[4] as such is inadequate for, unlike enzymes, the tumor agents essentially reproduce themselves and can be perpetuated by passage *in vivo*. It is on account of this fact that some investigators find difficulty in entertaining the conception that the agents may be inanimate.

Rather than connected with the cell metabolism, and contrary to the above conception, the tumor-producing mechanism in our opinion seems to be more closely related to the function which in the cell carries the fixed characters and controls the specific differentiation. The chicken tumor principle which affects a normal mesenchymatous cell forces it toward a further differentiation and its presence leads to the appearance of new functional and morphological characters. The most striking example is the transformation of a plain connective tissue cell of the breast region into a chondroblast or an osteoblast in the case of a transmissible osteochondrosarcoma. It is not easy to accept the notion that ordinary parasites, even endocellular agents, would induce such delicate, specific and, in fact, physiological evolution of the affected cell. Moreover, the induced characteristics are inherited by the daughter cells, as is also the property of transmitting the acquired characters to new cell generations. It is evident that in some way the system which in the cell is the carrier of individual specificity must be involved, and the association between the agent and the cell intimate enough to permit an evolution similar to that of existing cell types, integrant to the normal organism. This semi-physiological factor of differentiation and growth

[4] Murphy, in his original paper on the subject, used the term "enzyme-like" only to emphasize the conception that the agents were produced by cells. No closer likeness was suggested or considered.

is found in excess in developing tumor tissue, can be extracted in active form and, when brought in contact with normal susceptible cells, may be included in the cellular structure and is capable of reproducing faithfully the characteristics of the type.[5]

There are specific processes which probably furnish a close analogy to the phenomenon which controls the evolution of the tumor cell, namely, the substances which are responsible for the individual pneumococcus types, demonstrated *in vivo* by Griffith. Recently Alloway, in Avery's laboratory, has been able to extract the active principle from the cells. Here an agent present in a specific type of pneumococcus has the power to confer upon the undifferentiated cell the type specificity of the organism from which the agent was extracted. The phenomenon of heat-flocculation of Burnet furnishes another example of similar acquisition and transmission of specific characters. In this case, the Melitensis type of the Brucella group may acquire the property of the other type when grown in the presence of substances derived from the Paramelitensis organism. Here also the typical changes are inherited and transmissible to the new generations. Thus we have a group of agents, products of specialized cells, capable of conferring the peculiar type quality upon other cells of the same species, which in turn may produce the active factor and transmit it to their descendants. Wollman claims for the phenomenon of bacteriophagy a similar mechanism and suggests that the chicken tumors might be included in the class of disorders originating in growth disturbances designated by him as "paraheredity." There are other agents at present classed among the viruses which may likewise correspond to this type of mechanism. In the pneumococcus group there are three major types with three corresponding specific agents. There are at least seven distinct types of chicken tumors so far investigated, each possessing an agent extractable from the cell, capable of transforming the normal, undifferentiated elements into malignant cells with all the peculiarities of those supplying the agent.

Obviously the character, dominant in these pathological processes, is the acquisition of new specific properties and the transmission of inductive agents separable from the cells. The ability of such agents

[5] A discussion of the possible support given to this conception by the induction of tumors with factors derived from active normal tissue or with chemicals has been omitted. While these results are significant they have not been reproducible with sufficient regularity to be considered of theoretical importance at the moment.

to induce permanent, inherited changes in cells may justify the suggestion made by Murphy to differentiate them from the group of viruses by the restricted denomination of "transmissible mutagens."

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